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Evolution of *Streptococcus iniae* after vaccination and molecular underpinnings of capsular antigenicity

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Abstract

Streptococcus iniae causes severe septicemia and meningitis in farmed fish and is also occasionally zoonotic. Vaccination against *S. iniae* is problematic, with frequent breakdown of protection in vaccinated fish. The major protective antigens in *S. iniae* are the polysaccharides of the capsule, which are essential for virulence. Capsular biosynthesis is driven and regulated by a 21-kb operon comprising up to 20 genes. In a long-term study, we have sequenced the capsular operon of strains that have been used in autogenous vaccines across Australia and compared it with the capsular operon sequences of strains subsequently isolated from infected vaccinated fish. Intriguingly, strains isolated from vaccinated fish that subsequently become infected have coding mutations that are confined to a limited number of genes in the CPS (capsular polysaccharide) operon, with the remainder of the genes in the operon remaining stable. Mutations in strains in diseased vaccinated fish occur in key genes in the capsular operon that are associated with polysaccharide configuration (*cpsG*) and with regulation of biosynthesis (*cpsD* and *cpsE*). This, along with high ratios of non-synonymous to synonymous mutations within the CPS genes, suggests that immune response directed predominantly against capsular polysaccharide may be driving evolution in a very specific set of genes in the operon. From these data, it may be possible to design a simple polyvalent vaccine with a greater operational life span than the current monovalent killed bacterins.

I investigated antigenic effects of a key CPS biosynthesis gene, *cpsG,* a putative UDP-galactose 4-epimerase that has three sequence types based on the insertion or deletion of the three amino acids leucine, serine and lysine in the substrate binding site of the protein. To elucidate the role of *cpsG* in CPS biosynthesis and capsular composition, I first prepared isogenic knockout and complemented mutants of *cpsG* by allelic exchange mutagenesis. Deletion of *cpsG* resulted in changes to colony morphology and cell buoyant density, and also significantly decreased galactose content relative to glucose in the capsular polysaccharide as determined by GC-MS, consistent with epimerase activity of cpsG. There was also a metabolic penalty of *cpsG* knockout revealed by slower growth in complex media, and reduced proliferation in whole fish blood. Moreover, whilst antibodies raised in fish against the wild type cross-reacted in whole cell and *cps* ELISA, they did not cross-opsonise the mutant in a peripheral blood neutrophil opsonisation assay, consistent with reported vaccine escape. We have shown here that mutation in *cpsG* results in altered CPS composition and this in turn results in poor cross-opsonisation that explains some of the historic vaccination failure on fish farms in Australia.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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List of Abbreviations

CPS	Capsular Polysaccharide
EPS	Exopolysaccharide
ELISA	Enzyme Linked Immunosorbent Assay
TEM	Transmission Electron Microscopy
ST	Sequence Type
WT	Wild Type

Chapter 1

Introduction and Literature Review

“When we try to pick out anything by itself, we find it hitched to everything else in the Universe.”
- John Muir

1 Introduction

Foreign microbes in contact with the host's immune system are subject to defence mechanisms that are equipped to neutralise them. Adaptive or acquired recognition occurs via antibody responses to surface molecules or antigens on a foreign invader such as a pathogen. Pathogens are microorganisms that cause infection within the host, and may include resident bacteria if homeostasis is disturbed (1). Antigens on pathogenic bacteria are composed of proteins and/or carbohydrates (2), and can be found in the cell wall, capsule, and intercellular slime/mucous on bacteria. Numerous variations in antigen structure and composition allow the bacterium to evade host defences (2). Evolution to avoid the host's immune response is the driving force for antigenic variation and ultimately results in improved fitness of the bacterium in the immune host. This evolutionary process has been tracked during vaccine programs, with new strains re-emerging that are antigenically different to previous strains (3-5). The most striking examples of antigenic variation include *Streptococcus pneumoniae*, with over 90 different serotypes occurring (4, 6), and influenza virus, that is constantly changing in response to vaccine selection pressure (7).

Vaccination remains the most effective tool in the global fight against infectious diseases, yet each year new "pandemics" arise as new serotypes emerge, evidenced by antigenic variation of influenza virus in response to flu vaccine selection pressure (7). Every year, flu vaccines are reformulated to target the main circulating viruses (determined by world-wide surveillance) and their highly mutable antigenic structures (8). Vaccine efficacy is dependent on matching the antigenic structures of the influenza strains that are circulating through the population. In most cases, the vaccine matches adequately affording 70-90% reduced influenza cases, but may only be 40-60% effective when it matches less well (9).

Vaccines against bacterial pathogens are also subject to selective pressure of the host immune system. For example *S. pneumoniae* strains use molecular camouflage to avoid immune attack that is primed through vaccination (10-12). This camouflage may be achieved, in part, by changing the polysaccharide capsule (CPS) external to the bacterial cell wall. The CPS encapsulates bacteria, is highly antigenic, and the structure varies among not only bacterial species, but also between strains within a species (13). Targeting various CPS structures and trying to anticipate selective pressure from the host induced by vaccines has proven difficult (14, 15). Formulating vaccines has been

traditionally based on identifying proteins that elicit an immune response, purifying these, and testing using model organisms (8, 16). More recently, reverse vaccinology has been employed to identify a suite of potential vaccine candidates (17). Reverse vaccinology may hold the key to designing appropriate vaccines based on virulence factors identified in whole genome sequences. Since there are various factors at play in causing infection, a vaccine that targets multiple virulence determinants is ideal (18). At the same time, understanding the molecular mechanisms of the virulence determinants and how the host is responding is equally important.

1.1 Objective

Streptococcus iniae, a zoonotic bacterium, expresses several virulence determinants, with the CPS being most immune-dominant (19). A common aquatic pathogen, *S. iniae* is vaccinated against on aquaculture farms to prevent future outbreaks. However, like flu shots, these vaccines are only effective for a short time as the bacteria evolves and re-emerges (20). In order to develop effective vaccines, we therefore need to know how *S. iniae* is changing in response to vaccines and how these changes affect the antigenicity of this pathogen.

1.2 Aims

The aim of this project is to address one central question: how do specific mutations in the capsular operon of re-emergent strains of *S. iniae* affect antigenicity?

Specific aims:

1) *Identify re-emerging strains of S. iniae and determine genetic effects using large scale screening of capsular genes*

2). *Use allelic replacement to knockout genes in the capsular operon of S. iniae, and elucidate phenotypes with a defined genetic background*

3) *Raise antibodies in barramundi and use ELISA and killing assays to determine antigenic properties with mutant strains*

1.3 Study Setting

The work for this thesis was focused on re-emerging strains of *S. iniae* from barramundi (*Lates calcarifer*) aquaculture farms in Australia. Barramundi farms in Australia are unique in that they have a closed cycle for monitoring vaccine driven evolution by using autogenous vaccines. Strains from re-emerging infections with *S. iniae* were stored accordingly and analysed.

1.4 Overview of Thesis Chapters

Chapter 1 includes a concise literature review and background highlighting the need for antigenic studies in *S. iniae*.

Chapter 2 is the foundation for the thesis and its subsequent studies. It's the first time a large-scale genetic screen was performed on re-emerging *S. iniae* isolates in Australia. Specific biosynthesis genes were found to possess mutations, and were the target for further investigation into capsular antigenicity.

Chapter 3 looks at the first biosynthesis gene of interest *cpsG*, putatively an UDP-glucose 4'-epimerase. Amino acid mutations identified in *cpsG* are unique from the large screen found in chapter 2. In one strain an amino acid insertion of LSK (leucine, serine, lysine) and in another strain in the same location, a deletion of the same amino acid sequence. The strains were vigorously tested for enzyme activities, blood killing ability, and ultimately allelic replacement for total effect of the gene in the capsular operon.

Chapter 4 discusses conclusions and findings from Chapter 2 & 3.

1.5 Literature Review

1.6 Aquaculture

For centuries, humans have fished for food and this industry provides a major source of high quality protein throughout the world. As a consequence, many regions have witnessed over fishing of desirable food species coupled with a lack of timely stock recovery (21). A more sustainable answer has been the rapid growth of aquaculture, where aquatic organisms including plants, shellfish and fish, are farmed and sold to local, national, and international markets. Over the last few decades, aquaculture has expanded to farm fish that are typically exploited from overfishing, increasing production from 9% of total food fish in the 80’s to 43% of total fish for food use in 2012 (22).

There are several types of facilities for farming aquatic species, with the main goal being a product that is sustainable and profitable. Farm facilities are designed to mimic the natural habitat of the marine organism being cultivated (open ocean cages, ponds, coastal cages), however, with advances in technology, indoor re-circulating systems have become the preferred method with low environmental impact and very high potential yields (23). Indoor re-circulation systems work by passing water through filters where bacteria are used to breakdown the by-products formed by the organisms being farmed (23). Although expensive, this method allows more “control” in an otherwise highly variable setting. Outdoor ponds are less expensive and widely used, but are susceptible to environmental and anthropogenic factors that make it difficult to control infections (24). For these reasons, optimal animal husbandry is critical as well as water quality management to ward off infections.

1.6.1 Aquaculture infections and treatment options

As aquaculture became increasingly intensified, infections were more prevalent as fish were stocked at higher densities, enabling rapid proliferation and transmission between individuals. In the 1980’s and 1990’s, Norway’s salmonid industry was devastated by *Vibrio* and *Aeromonas salmonicida* infections (25).

This initially led to a surge in antibiotic use to control infections during the 1980's, but this strategy was replaced in the mid-1990's with vaccines, as antibiotic use in major aquaculture species had been severely compromised due to the rise in antibiotic resistance (25). There are three ways to administer vaccines: immersion, intra-peritoneal injection, and orally (26). Immersion vaccines are used in small fish but elicit a relatively short duration of immunity while oral vaccines have had little success in a practical farm environment due to difficulties with protecting high antigen doses through the gut (16). Intraperitoneal injections are frequently used as a mode for delivering vaccines because this elicits a high circulating antibody response. Although intraperitoneal injections are labour intensive they are the most commonly used form of vaccination in fish due to their high efficacy and long duration of immunity (26, 27). Intraperitoneal vaccines are usually comprised of formalin-inactivated bacteria, although occasionally live attenuated bacteria and recombinant proteins have been applied (22, 26, 27).

In Australian aquaculture producers have to approach infection prevention/treatment differently as there are few licenses for commercial vaccines. Aquaculture in Australia began in the 1960's, and is the fastest growing production sector in Australia (28). One of the more successful farmed fish species is barramundi (*Lates calcarifer*), grossing thirty million dollars annually in Australia (29). However, since 1992, infections caused by *S. iniae* in farmed barramundi (30) have caused serious problems for fish farmers, and thus treatment options have been intensively researched. Treatment with antibiotics such as penicillin, erythromycin, and carbenicillin were trialled in the Amazon river dolphin as an inexpensive way to treat *S. iniae* infection, but it was still inefficient as some treatment cycles take over 18 months (31). Autogenous vaccination is most often used on Australian barramundi farms to prevent *S. iniae* infection. Autogenous vaccines must be comprised of strains that are initially isolated from the farm where the infection occurred. Strains are documented through the state veterinary laboratories and then provided to a registered manufacturer to produce a custom vaccine, which may then be used to vaccinate stock for introduction onto the original farm (5). Autogenous vaccines (delivered via intraperitoneal injection) generally work well however, occasionally re-emerging strains occur in previously vaccinated stock due to high variability in the capsular polysaccharide (CPS) structure, the major antigenic determinant in *S. iniae.* This was first reported in rainbow trout in Israel, and latterly in Australian barramundi (19, 20). This poses a significant problem for development of effective vaccines against highly variable pathogens that are increasingly occurring in the growing finfish aquaculture industry.

1.7 *Streptococcus iniae* – a global pathogen

S. iniae is ubiquitous in warm and temperate waters, infecting wild and farmed fish including tilapia, rainbow trout, and barramundi (5). Gram positive and beta-haemolytic, *S. iniae* forms cocci chains, like other streptococci, and generally displays small translucent colony morphology (5). *S. iniae* was originally isolated from an Amazon freshwater dolphin in 1976 (32), and has a wide host range, including humans (5). There is no definitive serotyping scheme for *S. iniae* although several reports of differing "serotype" exist: Serotype I, considered "classical", was reported to result in less severe pathogenesis compared with some other serotypes (33). Infection with "serotype I" in fish, results in lesions on the skin and rarely meningitis. "Serotype II" differed biochemically from "serotype I" being arginine dihydrolase and ribose positive, and was identified after a vaccine program in Israel (34). Pathogenesis includes damage to different organs, crossing of the blood brain barrier, and *S. iniae* has also been found in the bone of the fish host (20, 33, 34). Infections in humans can occur when an open wound is infected with *S. iniae* during the handling or processing of infected fish (5). Symptoms can range from bacteraemic cellulitis, arthritis, toxic shock and sepsis (35). These symptoms are exacerbated in immunocompromised individuals, and are often hard to treat as the bacteria enter the bloodstream (36).

In fish, *S. iniae* causes skin lesions, exophthalmia or eye bulging (usually bilateral), meningitis, and in most cases leads to death (35). Again, severe infections occur in fish when the immune system is compromised. Fish become immunocompromised due to fighting with other fish (which stresses individual fish), inadequate water quality conditions, or poor fish husbandry (5). Transmission of *S. iniae* in fish is not well understood, but in marine caged fish is thought to occur via an oral-faecal route or by ingesting carrier fish (37, 38). While most infections of *S. iniae* in Australia occur in pond aquaculture, the environmental source of the pathogen remains unknown (38). Understanding how *S. iniae* is transmitted, and more importantly where the bacterium comes from, is critical for vaccine design, infection control, and evolution of re-emerging strains.

1.7.1 *S. iniae* virulence factors

Progress has been made in the identification of factors that contribute to *S. iniae* virulence (10, 19, 35, 39, 40). Genomic studies have shown homology to *Streptococcus pyogenes* genes found including M-like protein (*simA*), C5a peptidase (*scpl*), and genes of the *sag* operon that contribute to SLS production (41, 42). Mutation of the genes encoding these proteins resulted in attenuation of *S. iniae* virulence, as well as changes to pathology at

the site of infection (41, 42). In light of these findings that M-like protein contributes to virulence, a vaccination and challenge experiment was designed in barramundi (Lates calcarifer) using M-like protein (*SiMA*). However, the vaccine did not protect against challenge strains (43) Other virulence determinants have been identified on the surface of *S. iniae* (Fig. 1), and some have subsequently been tested as vaccine candidates (44). Unfortunately, none of these experimental vaccines is able to elicit cross protection, reinforcing the view that that the CPS is the major antigenic determinant (35, 43).

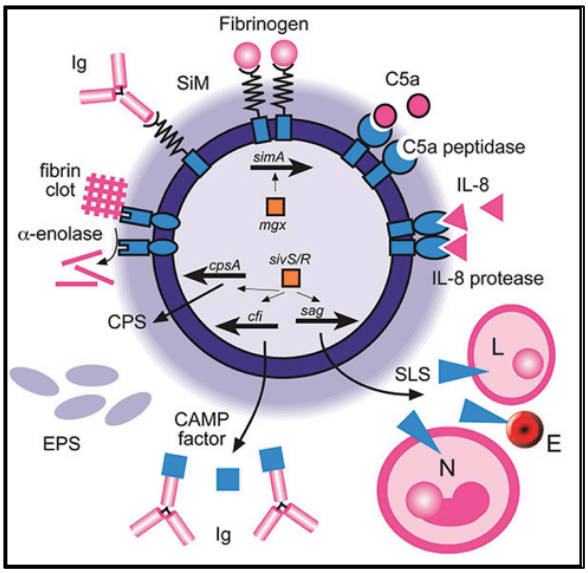

Fig. 1 *Streptococcus iniae* virulence factors that may protrude through the polysaccharide capsule, or are secreted (Baiano & Barnes 2009). Abbreviations: C5a = complement component 5a, IL-8 = interleukin 8, L=Lymphocytes, E= Erythrocytes, N= Neutrophils, SLS= Streptolysin S, Ig= Immunoglobulin, CAMP= Christie, Atkins, and Munch-Peterson), EPS= extracellular polysaccharides, CPS= capsular polysaccharide, SiM= *Streptococcus iniae* M-like protein.

In spite of this progress, the majority of vaccines against *S. iniae* are still simple formalin inactivated cultures that result in the immune system substantially targeting the polysaccharide capsule. The identification of the capsular operon in *S. iniae* was a significant finding, and by using molecular techniques, has advanced understanding of both virulence and immune evasion. For now, the hurdle remains to find a suitable vaccine that will provide cross protection and not give rise to new emerging strains.

1.8 Vaccine escape and the polysaccharide capsular operon

1.8.1 Evolution of new serotypes

Several studies have shown pathogens have the ability to re-emerge after vaccine programs (45-48). In the 1980's vaccines were initiated for infectious bursal disease (IBD) in chickens, which causes respiratory infections and contributes to economic loss globally (47). Antigenically distinct strains started emerging in the U.S., and across Europe, with highly virulent strains dominating the population and mortality rates at greater than 60 % (49). Similarly, avian influenza, widespread amongst commercial poultry, has faced difficulty with strains re-emerging after vaccination programs that targeted specific antigenic determinants (50). Animals are not the only species affected by re-emerging strains, human pathogenic strains have also evolved due to clinical intervention. During the 80's a vaccine containing specific recombinant surface antigens was designed for patients with Hepatitis B (51). Mutations were found in subsequent strains with amino acid modifications to specific surface antigens (48). These studies all share a common issue of epitope escape mutants, or serotypes that arise in a population displaying differences in antigen presentation. Most vaccines against pathogens are designed to target protective antigens or epitopes that are highly immunogenic, as such they are usually the most variable (52). In anticipation of epitope variation, vaccines are often designed with multiple recombinant antigens, as in the case with Hepatitis B amongst others, that are known to illicit an immune response (51). This design leaves an opening for non-vaccine strains to gain, overtime, components not present in the vaccine to elude the immune system (47). Alterations to epitopes are not the only type of vaccine driven evolution, virulence adaptations have also been described (15). Virulence adaptations can occur when vaccines are designed to reduce pathogen growth, toxicity effects, and transmission (15). More specifically, how virulence interacts with vaccinated and un-vaccinated hosts over time. This type of vaccine driven evolution has attracted attention recently, as Read and colleagues were able to show that vaccinations with "leaky vaccines" (where transmission is not terminated) against Marek's disease virus (MDV) in commercial chickens led to unvaccinated chicken infections and ultimately hyperpathogenic strains of MDV (55). It is important to distinguish between these types of vaccine driven evolution and how serotypes can arise in a population however, the focus here will remain on epitope variation driven by vaccination in streptococcal infections.

1.8.2 Streptococcal infections and re-emerging strains

There are several different streptococcal species that infect a wide variety of hosts (5, 12, 37, 56, 57). Many streptococci also live commensally, only causing infection in immunocompromised individuals. These include *Streptococcus pyogenes*, more commonly referred to as "strep throat", and *Streptococcus agalactiae* (GBS), that may live commensally within a percentage of the population, occasionally causing overt infectious disease (12, 57). In spite of the diversity in host range, and modes of infection/colonisation, there are several similarities among streptococcal virulence factors that allow them to evade host defences, the most ubiquitous being the capsule (11, 19, 58). Vaccine programs using purified CPS have shown promise for short-term infections, however, several re-emerging cases have been reported with evidence of epitope variation (45, 59). *S. pneumoniae,* well known for infecting humans globally, possesses over 90 capsular serotypes (47). Re-emerging strains were identified after clinical trials during the 1990's, where vaccines were composed of multiple capsular serotypes. These types of changes to the CPS have also been documented in GBS after vaccine programs (59). Surprisingly, a capsular shift occurred in a usually homogenous GBS strain due to an exchange of a DNA fragment in the cps operon, subjecting the vaccine target to variation (59). One of the first cases of a re-emerging *S. iniae* strain was in Israel after a vaccination program, and subsequently *S. iniae* strains were identified after vaccination programs in Australia (3, 20). The Israeli vaccine program was performed on rainbow trout (*Oncorhynchus mykiss*), and re-emerging strains of *S. iniae* had evolved to produce excessive amounts of highly antigenic EPS (extracellular polysaccharide) (3). This led to increased virulence and enhanced the ability of the pathogen to escape the immune system and establish infection. Re-emerging strains of *S. iniae* identified in Australia were found in barramundi (*Lates calcarifer*), on several different fish farms with vaccine programs. In some of the re-emerging Australian *S. iniae* strains, the CPS was not present and a new, unusual pathology was present in fish (20). These strains were found to infect the bone of fish, causing severe spinal deformities but low mortality. Where as other re-emerging strains were infecting the previously vaccinated fish, but the CPS yielded differences in amino acid sequence when compared to the WT strain. Sequencing of the CPS operon from all re-emerging strains in Australian fish farms revealed that vaccination was driving mutation of key genes involved in polysaccharide biosynthesis and transport. SNP's and truncated genes were identified in the CPS operon, contributing to re-infection and the unusual pathology that was observed with the loss of capsule (20).

1.8.3 CPS operon

The polysaccharide capsule of *S. iniae* is similar to the CPS found in other virulent streptococcal groups and bacterial species in general (11, 13, 60). The CPS is covalently bound to the surface of the bacterial cell, composed of monosaccharides, and may contain several configurations depending on the number of hydroxyl groups present (19, 61). The capsular operon encodes the machinery that synthesises, exports and anchors the polysaccharide to the cell. Clustering of CPS genes is a common feature among most bacterial species, spanning a range from 10 – 20 kb in length (62). Genes within the 21-kb capsular operon of *S. iniae* (Fig 2.) were identified in 2007 (19), and genes of interest were identified as those that are putatively responsible for initiating polysaccharide biosynthesis (*cpsY*, and *cpsD-cpsH*), which are conserved among several streptococcal species (36). Using allelic replacement, mutation of the gene encoding an auto-phosphorylating protein tyrosine kinase, *cpsD,* resulted in diminished phagocytic function due to a lack of capsule production (10). *cpsY*, a transcriptional regulator, was found to contribute to protection of *S. iniae* against neutrophil killing and regulate growth *in vitro* (63). Two other genes that are implicated in capsule biosynthesis that have not been previously investigated are *cpsE,* a putative glucose dependent glycosyltransferase and *cpsG*, a putative UDP-glucose-4'epimerase. Glycosyltransferases build monosaccharide moieties that form branched or linear glycan chains (64). Antigenic variation in the glycoslytransferase operon of *Salmonella enterica* subspecies Typhimurium, showed an increase in sugars on the O-antigen structure ultimately changing the serotype (65). UDP-glucose 4' -epimerases control the ratio of glucose:galactose as part of the last step in the Leloir pathway for galactose metabolism, and is found in all three branches of life (66, 67). Mutations of this gene are known to cause galactosemia in children and affect their ability to metabolise Leloir products (68). These genes play an important role in various species reinforcing the need to study them in *S. iniae*. Polysaccharide biosynthesis genes play a critical role in the final capsular product, determining CPS antigenicity, and how the bacterium eludes its host (5, 36, 63).

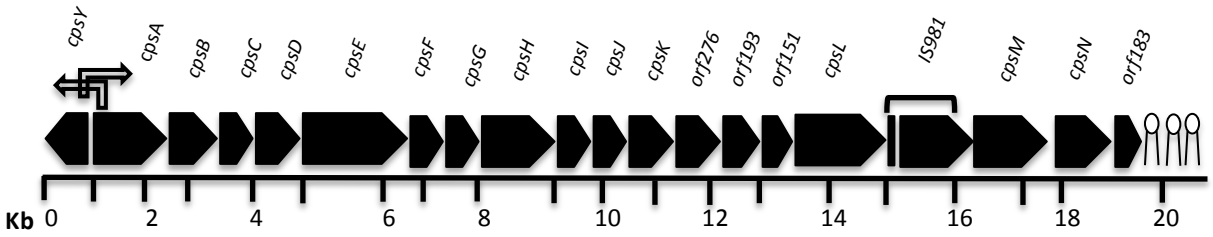

Fig. 2 Genes and their orientation in the 21kb capsular operon of the *S. iniae* type strain (140). (Figure modified from Lowe et al., 2007)

Although molecular differences exist between bacterial species, the capsule is required for successful infection by many systemic pathogens. Intriguingly, type III group B *Streptococcus* (GBS) species have a different chemical composition in their capsule via sialic acid residues, thus preventing complement C3 deposition on the bacterial cell (58). Adaptation and evolution of pathogens reveals the ability to alter their capsules and prevent immune detection (3, 40).

Studies on the composition and chemical configuration of *S. iniae* CPS are lacking, but we know the CPS is a complex matrix of neutral and charged sugars (10, 39). Polysaccharide capsules mask the pathogen allowing them to successfully by-pass the first line of defence and disseminate through the host. Without the capsule, the immune system has a better chance of recognising surface structures and eliminating the pathogen (11, 36).

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Evolution of the capsular operon of *Streptococcus iniae* in response to vaccination

Running title: Capsule evolution in *S. iniae*

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Abstract

Streptococcus iniae causes severe septicaemia and meningitis in farmed fish and is also occasionally zoonotic. Vaccination against *S. iniae* is problematic with frequent breakdown of protection in vaccinated fish. The major protective antigens in *S. iniae* are the polysaccharides of the capsule, which are essential for virulence. Capsular biosynthesis is driven and regulated by a 21 kb operon comprising up to 20 genes. In a long-term study, we have sequenced the capsular operon of strains that have been used in autogenous vaccines across Australia and compared them with the capsular operon sequences of strains subsequently isolated from infected vaccinated fish. Intriguingly, strains isolated from vaccinated fish that subsequently become infected have coding mutations that are confined to a limited number of genes in the *cps* operon with the remainder of the genes in the operon remaining stable. Mutations in strains in diseased vaccinated fish occur in key genes in the capsular operon associated with polysaccharide configuration (*cpsG*) and with regulation of biosynthesis (*cpsD* and *cpsE*). This, along with high ratios of non-synonymous to synonymous mutations within the *cps* genes suggests that immune response directed predominantly against capsular polysaccharide may be driving evolution in a very specific set of genes in the operon. From these data it may be possible to design a simple polyvalent vaccine with a greater operational lifespan than the current monovalent killed bacterins.

2.1 Introduction

Evasion of the adaptive immune response through genetic evolution of a pathogen is a major factor governing long-term vaccine success (15). This is dependent on the nature of the antigens targeted by the adaptive immune response: If they are highly conserved, the diversity among the population will be low, or the population may be eliminated altogether with re-emergence of disease in vaccinated stock rarely occurring (15, 16). In contrast, adaptive immune selection against highly polymorphic antigenic determinants may result in pathogen populations restructuring into antigenic types or serotypes, with certain types predominating in vaccinated stock and fluctuations in the genetic structure of the population dictated by the serotype or types included in the vaccine (15).

Vaccinations against *Streptococcus iniae* infections in barramundi farms in Australia provide an ideal model for exploring evolution of pathogen populations. Firstly, it is almost impossible to eliminate the source of infection from the farm. In ponds and marine cage systems reinfection from sediments, wild fish and other aquatic inhabitants is unavoidable (1, 7, 8). Even in completely enclosed recirculating systems, economics dictate that the system cannot be shut down and disinfected completely, thus populations of the pathogen are likely to remain in biofilms on the pipe work, tanks and filters. Secondly, the infectious agent, *S. iniae* is highly variable (6, 12, 26), with its major antigenic determinant, polysaccharide capsule (6, 18, 21), being highly polymorphic: Novel capsular serotypes have already led to vaccine failure in fish farms in several parts of the world, including Australia (1, 4, 11, 26). Finally, in Australia, there is no licensed generic vaccine against *S. iniae* so autogenous vaccines ('custom' vaccines prepared from an isolate taken directly from the original farm and only used on that farm) are routinely employed to prevent streptococcosis. This creates a closed cycle whereby a pathogenic strain isolated from a particular farm is used to prepare a vaccine that is then used to vaccinate stocks that are reintroduced onto the same farm.

The capsular polysaccharide (CPS) is located on the outermost layer of bacterial cells and are ubiquitous across several bacterial species (28). For pathogenic *Streptococcus* species, CPS varies depending on serotype and is a recognized virulence factor that contributes to immune evasion (10, 18). In Group B *Streptococcus* (*Streptococcus agalactiae*), variations in the polysaccharide capsule have been implicated in reduced complement C3b binding, and ultimately avoidance of phagocytosis (19). Similarly, research on *Streptococcus pneumoniae* revealed that morphological changes in the

capsule occur in order to adapt to the host's environment (13). In *Streptococcus iniae*, as with the other streptococcal species, production of the capsular polysaccharide appears to be necessary for infection (21). Locke and colleagues (2007) elegantly demonstrated that *cpsD,* a homologue of a gene shown to be required for CPS production in Group B *Streptococcus* and *S. pneumoniae*, was required for capsule formation and export in *S. iniae*. Although a few studies have investigated virulence factors in *S. iniae*, the function of the genes in the capsular operon are less well known, and how these translate into multiple serotypes in this species is yet to be elucidated.

In the present study, we investigated molecular evolution of the capsular operon of *S. iniae* from diverse origins in order to determine which genes were most variable. We found that five genes out of the approximately 21-kb *cps* operon were highly variable, and when we investigated case studies of repeated autogenous vaccination and vaccine failure at Australian barramundi farms for links between the highly mutable genes and reinfection of vaccinated stock, we found a direct correlation with the variable *cps* genes and vaccination failure. Surprisingly, no capsule was formed in some of our isolates, yet the pathogen was still able to infect the host, albeit with a completely different pathology. To further understand these findings on the effect on vaccination against *S. iniae*, we examined serological cross-reactivity using antibodies raised in fish between isolates with differing *cps* gene sequences from several of the case studies in which vaccinated stock became reinfected. Our results suggest that polyvalent vaccines comprising different *cps* sequence types are partially effective but that future generic vaccines may need to target alternative antigens that are less polymorphic than CPS.

2.2 Materials and Methods

Bacterial strains and culturing

Bacterial isolates received from culture collections or direct from fish farms or veterinarians were stored at -80 °C in Todd-Hewitt broth (THB) containing 20% glycerol until required, and are listed in Supplementary Table 1. Strains were recovered from stock without defrosting and grown on Columbia Agar base containing 5% defibrinated sheep blood (Oxoid, Australia) at 28 °C for 24-48 h. Where isolates were obtained from farm cases through veterinarians, histopathology was performed by the veterinary laboratories using standard techniques. Identity of strains was confirmed by diagnostic PCR as previously described (20, 27), and by sequencing the 16S rRNA gene (26, 27).

DNA extraction, primer design, PCR, and sequencing

S. iniae genomic DNA was extracted from freshly-grown cells using an enzymatic lysis method as previously described (27a). Primers for PCR and sequencing were designed using the *S. iniae* capsule operon sequence available on GenBank (sequence accession number AY904444). The primers used were as described in Barnes (5) and are listed in Tables S2 and S3 in the supplemental material.

Capsular operon genes were amplified individually or in blocks of several genes (see Table S2 in the supplemental material) with a proofreading DNA polymerase (PrimeStar HS DNA polymerase, Takara, Japan) in 25 µL reactions composed of: 5 µL of 5 x PCR buffer, 0.5 µL of 4 x 2.5 mM dNTP's, 100 ng of each primer, 0.15 µL of PrimeStar DNA HS DNA polymerase, 100-200 ng of extracted bacterial DNA, and the balance made up of sterile Milli-Q water. Reactions were carried out at annealing temperatures appropriate to each primer pair (see Table S2 in the supplemental material) (5). A "hot start" technique was employed to reduce the likelihood of non-specific amplification products prior to cycling as follows: 2 min at 94 °C for one cycle, followed by 35 cycles of denaturation for 15 s at 94 °C, 30 s at an appropriate annealing temperature, and extension at 72 °C of 1 min for every expected kb of amplicon. Reaction mixtures were analysed by agarose gel electrophoresis.

Where a single amplicon was produced, 0.6 µL of PCR product was added to 0.3 µL of a 1:2 mixture of Exonuclease I and Shrimp Alkaline Phosphatase and 3.1 µL of sterile Milli-Q water and incubated at 37 °C for 30 min followed by heating at 85 °C for 15 min before being sent for DNA sequencing by the Australian Genome Research facility (AGRF,

Brisbane, Australia). Where more than one amplicon was observed (rarely), they were excised from the gel with a sterile scalpel blade and then extracted from the gel slice using a commercial gel purification kit for subsequent sequencing (MEGA-Spin Gel Extraction Kit, Intron Biotechnology, Korea).

Chromatograms were analysed using Sequencher V 4.9 (Genecodes). Contigs were assembled using equivalent genes from the published genome as references and the assembled complete cDNA sequences were compared to the reference sequences using both nucleotide and translated variance tables in Sequencher 4.9. Sequence Types (STs) were assigned on an *ad-hoc* basis as discovered, with the sequence types for the type strain assigned ST1. Changes resulting in an amino acid change were assigned ST numbers while synonymous nucleotide changes were denoted with a letter appended to the identical amino acid sequence type. Sequences for each of the STs of *cps* genes were uploaded to GenBank as follows: *cpsY* ST1 and ST2, GenBank accession numbers JX164243 and JX164242, respectively; *cpsD* ST1, ST2, ST3, ST3A, and ST4, GenBank accession numbers JX164238, JX164245, JX164246, JX164239 and JX164247, respectively; *cpsE* ST1, ST2, ST3, ST4, ST5A, ST5, ST6, ST7, and ST8, GenBank accession numbers JX164231, JX164248, JX164232, JX164233, JX164249, JX164234, JX164235, JX164236, JX164237, and JX164250, respectively; *cpsG* ST1, ST2, and ST3, GenBank accession numbers JX164240, JX14244, and JX164241, respectively; and *cpsH* ST1, GenBank accession number JX181784.

Buoyant density assays

As an estimate of quantity of CPS expressed by differing strains, buoyant density was determined in continuous Percoll gradients as described previously (17). Briefly, a standard isotonic Percoll solution was prepared by mixing 9 parts Percoll with 1 part 1.5 M NaCl. Mid exponential phase and stationary phase THB cultures of each strain were washed in PBS and resuspended to an optical density (600 nm) of ~2.5 and a 0.5 mL aliquot was layered onto the Percoll. All gradients were centrifuged simultaneously in an Eppendorf 5518E refrigerated centrifuge at 4000 x *g* for 90 min with no brake. The experiment was repeated using new cultures at least twice.

Serological cross-reactivity by a whole-cell enzyme-linked immunosorbent assay (ELISA)

Lates calcarifer (barramundi) weighing 35 g were obtained from a commercial farm and held in 300 L round plastic tanks with aerated brackish (5 ppt) water at 30 ± 1 °C connected to a recirculating system. Water quality was maintained with mechanical and

biological filtration, checked daily and water changes were conducted when required. The fish were acclimated for 7 days and were fed to satiation with commercial pelleted diet (Ridley Aqua Feeds, Narangba, Australia).

Fish were anaesthetised with Aqui-S (Aqui-S, Lower Hutt, New Zealand) in accordance with the manufacturer's instructions then vaccinated by intraperitoneal (i.p.) injection with 100 µL of oil-adjuvanted (1:1 emulsion in Freund's incomplete adjuvant) formalin killed bacterin. Control fish were injected IP with PBS/adjuvant emulsion. Fish were allowed to recover in clean aquarium water before returning them to their respective tanks.

A total of 900 degree days postvaccination (i.e., 30 days at 30 °C), the fish were euthanized by lethal overdose of anaesthetic (Aqui-S) and bled by caudal venipuncture. Blood samples were allowed to clot at 4°C overnight and were centrifuged at 6000 x *g* for 15 min to collect serum that was then stored at -20 °C for subsequent analysis. The specific antibody response of each individual vaccinated fish was determined using enzyme-linked immunosorbent assay (ELISA) as described previously (9) with modifications. Microlon flat-bottom 96-well ELISA plates (Greiner) were coated by evaporating 100 µL/well of formalin inactivated suspension (OD_{600} = 1.0) of QMA0177, QMA0191 in coating buffer (carbonate-bicarbonate buffer [Sigma, Australia]) with a hair dryer. Coated plates were washed 3 times with tris buffered saline containing 0.1% Tween 20 (TBST) and blocked with 2% normal goat serum in TBST at room temperature for 1 h. The plates were washed 3 times with TBST and 100 µL primary antisera from vaccinated fish and control serum from sham vaccinated siblings was diluted 1:4 in TBST and incubated for 2 h at room temperature. Wells were washed 3 times with TBST and secondary antibody (monoclonal mouse anti-barramundi IgM, diluted 1: 32-fold in TBST [Aquatic Diagnostics, Stirling, United Kingdom]) was added and incubated for 1 h at room temperature. Wells were washed three times with TBST before addition of tertiary antibody conjugate (polyclonal goat anti-mouse IgG alkaline phosphatase conjugate, diluted 1: 30,000 in TBST [Sigma Aldrich]) and incubated for 1 h at room temperature. Wells were washed twice in 0.1% TBST and once in TBS, then colour was developed for 1 h using p-Nitrophenyl phosphate liquid substrate (50 µL/well) (Sigma Aldrich). Absorbance was measured at 405 nm with a Fluostar Optima spectrophotometer/fluorimeter/luminometer (BMG LabTech).

2.3 Results

Variation in capsular genotype is confined to a few genes in the operon

Capsular biosynthesis in *S. iniae* is under the control of a 21-kb operon containing around 20 genes. The operon has been fully sequenced (21) and characterized to some degree using site directed mutagenesis (18). To determine whether genetic changes in the capsular operon correlate with infection in vaccinated animals and virulence, we initially sequenced all genes within the capsular operon of 10 *S. iniae* isolates. These strains came from diverse origins and included strains from documented vaccine failures (4, 5, 26). Sequences of the genes from the capsular operon of these 10 isolates resulted in a number of surprising findings (Table 1). First, the number of synonymous (noncoding) mutations across the 21-kb operon was incredibly low, compared to coding mutations, which occurred frequently. Coding mutations, resulting in amino acid changes in the expressed proteins, were restricted to a limited set of genes within the operon. That is, the mutations were not evenly distributed across all of the 21 kb but were confined to a limited set of 5 genes, *cpsY, cpsD, cpsE, cpsG,* and *cpsH* (Table 1).

Table 1 Sequence types of genes from the capsular operon of 10 isolates of *S. iniae* separated either geographically or serologically[a]

Gene	ST for each strain no. (origin)									
	QMA0072 QLD	QMA 0076 QLD	QMA 0083 WA	QMA 0140 USA	QMA 0155 NSW	QMA 0165 QLD	QMA 0177 NT	QMA 0191 NT	QMA 0188 ISR	QMA0186 ISR
cpsY							2	2		
cpsD	3	3	3		3	3	3	3	3A	2
cpsE	3A	4	3		3	3A	3	3	2	2
cpsG			2		2		3		X	X
cpsH						2				

[a] Gene sequences from the type strain (QMA0140) were designated ST1. As coding mutations were discovered, they were allocated a new sequence type number arbitrarily in the order of discovery. Non-coding mutations are given the ST number of the identical expressed sequence and identified as variants with a letter (eg. ST3A expresses the same protein as ST3 but has one or more non-coding single nucleotide polymorphisms [SNPs]). Genes are ST1 if not otherwise indicated. X indicates a deleted gene. Only genes that differ from the type strain are included in the table. Strain geographic origin abbreviations: QLD, Queensland, Australia; WA, Western Australia; NSW, New South Wales, Australia; NT, Northern Territory, Australia; USA, United States of America; ISR, Israel.

Subsequently, we extended our study to an additional 29 isolates and sequenced blocks of genes encompassing these regions (Table 2). We found that *cpsE* had the largest diversity (that is, the largest number of variant sequence types [STs]). However, *cpsE* was not the most frequently modified gene in the operon; variance in *cpsG* occurred more frequently in Australian isolates (see Table S1 in the supplemental material) (5). *cpsD* was also variable, with 4 different coding sequence types found among the isolates sequenced. In contrast, diversity of *cpsD* was lower in Australia, with only two sequence types found.

Table 2 Nucleotide and amino acid changes associated with differing *cps* gene sequence types

Gene and position	Nucleotides/amino acids in[a]:							
	ST1	ST2	ST3	ST4	ST5[b]	ST6[b]	ST7	ST8
cpsY								
535/179	TTC/F	TCC/S						
cpsD								
367/123	CCA/P		CTA/L					
385/129	TTA/L							
475/175	GTG/V	TTG/L	TTG/L					
cpsE								
119/40	GAA/E	GGA/G	GGA/G	GGA/G		GGA/G	GGA/G	GGA/G
136/46	TTT/F		GTT/V					
1426/476	CGT/R			CTT/L				
1081/361	CAT/H				Del C→	Del C→		
1108/370	TTA/L				TAA/stop	TAA/stop		
1096/366	AAG/K						ACG/T	
1315/439	GCA/A							GTA/V
cpsG								
495/165	TTA/L	Del TTA/L						
498/166	TCA/S	Del TCA/S						
501/167	AAG/K	Del AAG/K						
504/167.1			Ins TTA/L					
507/167.2			Ins TCA/S					
510/167.3			Ins AAG/K					
cpsH								
669/223	TTA/L	TTT/F						

[a] Underlining indicates the changed nucleotide relative to the type strain (ST1). Del, deletion; ins, insertion.

[b] A single nucleotide deletion leads to a changed amino acid sequence from aa 361 to an early termination at aa 370.

Indeed, ST3 was universal across all states in Australia, and ST1 was identical to the type strain from dolphins, arising only under high selective pressure in two cases in New South Wales and South Australia (see Table S1 in the supplementary material; see also the case histories described below). The *cpsY* gene also had low variability; all isolates examined carried ST1, regardless of global geographic origin with the exception of the isolates from Northern Territory, Australia, that, without exception, carried the ST2 *cpsY* variant (Table 2).

Coding mutations in the capsular operon correlate with autogenous vaccine failure

Reoccurrence of infection by *S. iniae* in vaccinated fish has been reported previously in Israel (4), and this appeared to be a result of changes in capsular polysaccharide and secreted exopolysaccharide (11). We sequenced the complete capsular operon in two postvaccine strains from Israel that had differing random amplified polymorphic DNA (RAPD) profiles (4) but that were apparently serologically cross-reactive (4, 11) and found a single coding mutation in *cpsD* (Table 1) resulting in a change from proline to leucine at amino acid (aa) position 123.

Northern Territory, Australia

In April 2005, streptococcosis emerged as a cause of high mortalities in farmed barramundi in Port Hurd, Northern Territory (NT), Australia. In July 2005, a cohort of fingerlings was vaccinated with an autogenous vaccine prepared from the isolate obtained from the index case and designated strain QMA0191 in the UQ strain collection (Table 3) and introduced onto the site. Further cases of clinical streptococcosis were recorded in the fish at Port Hurd in July, August and September, as well as isolated from brain and kidney in subclinically infected fish. All these cases were in nonvaccinated fish and are represented by strain numbers QMA0142, QMA0150, and QMA0153 (Table 3). In July 2006, an outbreak of clinical streptococcosis occurred in multiple cohorts of fish across the farm, including fish vaccinated with the autogenous vaccine containing strain QMA0191. An isolate taken at this time was also deposited in the UQ collection and designated QMA0177. Analysis of the capsular genotypes of the strains recovered from unvaccinated fish during 2005 indicated a consistent molecular serotype identical to the strain (QMA0191) used to produce the autogenous vaccine and explains the efficacy of the vaccine (Table 3). When disease arose across the site in July 2006, more than 6 months after vaccination, mortality resulted from strains with a coding change in *cpsG,* a 6-amino-acid (2 x LSK repeat) insert.

TABLE 3 Case histories of strain isolation and vaccination in three farms, a sea farm in Northern Territory and recirculating aquaculture farms in South Australia and New South Wales[a]

Location	Strain	ST of:			Isolation date (mo/yr)	Dates used in vaccines
		cpsD	*cpsE*	*cpsG*		
Northern Territory	QMA0191 (v)	3	3	1	04/2005	2005-2006
	QMA0142	3	3	1	07/2005	
	QMA0150	3	3	1	08/2005	
	QMA0153	3	3	1	09/2005	
	QMA0177	3	3	3	07/2006	
South Australia	QMA0160 (v)	3	3	1	12/1999	2004-2009
	QMA0159	3	3	2	03/2006	
	QMA0243	3	3	2	05/2006	
	QMA0244, QMA0245	3	3	2	10/2008	
	QMA0246+,QMA0247,QMA0248	3	3	2	03/2009	March/April 2009 divalent
	QMA0249	1	5*	X	05/2009	
New South Wales	QMA0155 (v)	3	3	2	12/2005	Early 2006 Monovalent
	QMA0220+	3	3	2	08/2006	2007-2008
	QMA0250+	3	3	2	11/2007	January 2008
	QMA0251+, QMA0252+	3	3	2	06/2008	July 2008 divalent
	QMA0233	1	5A*	X	11/2008	
	QMA0253+, QMA0254+	1	5A*	X	01/2009	March 2009 divalent
	QMA0236	1	5A*	X	03/2009	

[a] Numbers indicate sequence types of indicated *cps* genes. X indicates a gene deletion. All other genes in the *cps* operon were identical among strains in the same table unless otherwise indicated. *, strain had genes *cpsF-cpsM* deleted; (v), strain used in initial autogenous vaccine; +, isolate used in subsequent vaccinations.

South Australia

Vaccination of barramundi in a tank-based aquaculture facility in South Australia (SA) commenced in late 2004 using an autogenous vaccine prepared from an isolate from an outbreak in December 1999 (QMA0160). A subsequent outbreak in May 2006 resulted from a new strain (QMA0243) and again in October 2008 (QMA0244, QMA0255) continuing through the southern hemisphere summer to March 2009 (QMA0246 to QMA0248), with all isolates exhibiting a shift in amino acid composition in the *cpsG* (ST1 and ST2) genes (Table 3). A new autogenous vaccine incorporating both strain types was used to vaccinate new stock in March to April 2009. In May 2009, low-level mortality was detected in vaccinated stock, but symptoms were atypical; barramundi developed spinal fractures. In some vaccinated fish, these spinal fractures were colonized by Gram-positive cocci; perpetuating spinal inflammation (Fig. 1).

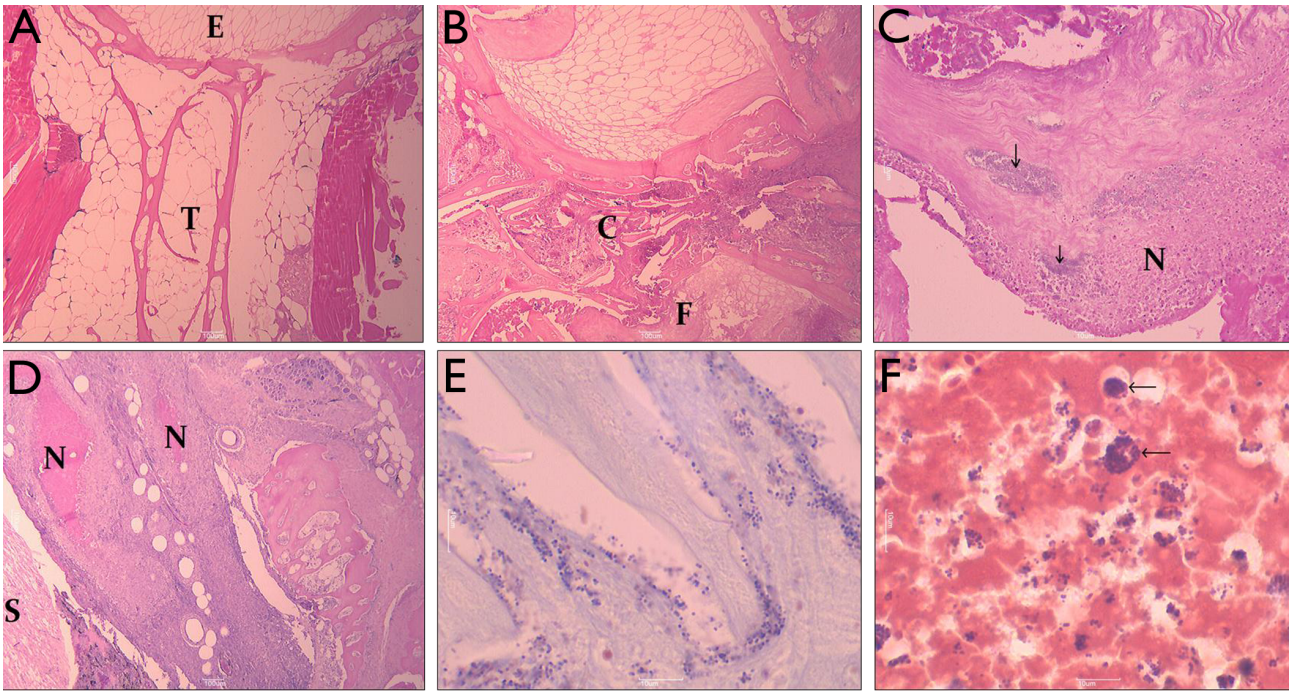

FIG 1 Histopathology of spinal inflammation in infected barramundi. (A) *L. calcarifer* sagittal spinal vertebral section showing normal structure of central vertebral spongy bone trabeculae, indicated by T, and endplate, indicated by E, at X40 magnification (hematoxylin and eosin [H&E]). (B) *L. calcarifer* spinal sagittal section at the point of collapse, indicated by C, of the intervertebral disc space due to inflammatory infiltrate and fracture of the vertebra, indicated by F, at X40 magnification (H&E). (C) *L. calcarifer* spinal sagittal section showing multiple colonies of Gram-positive cocci. Arrows indicate inflamed and necrotic tissue exudate, indicated by N, at the site of spinal fracture at X200 magnification (H&E). (D) *L. calcarifer* spinal sagittal section showing a severe intraspinal canal infiltration with inflammatory cells, caseous necrotic foci, indicated by N, containing colonies of phagocytosed coccoid bacteria. Inflammatory exudate is in close proximity to the spinal cord, indicated by S, at X40 magnification (H&E). (E) Gram stain of an *L. calcarifer*

sagittal spinal section showing colonies of Gram-positive coccoid and chain-forming bacteria in close association with the vertebral bony surfaces at X1,000 magnification. (F) Gram stain of an *L. calcarifer* spinal vertebral inflammatory exudate showing multiple Gram-positive coccoid bacteria. Note the potentially phagocytosed bacteria in cells (arrows), but there are also free bacteria in the exudate, at X1,000 magnification.

The brain and head-kidney (pronephros) of nine fish were sampled for bacteria, but no *S. iniae* was recovered, nor was *S. iniae* observed in the kidney during histology. *S. iniae* was only observed in, and recovered from, only the bone lesions and the resulting exudate (Fig. 1). The isolates that caused this unusual pathology were found to have a frameshift mutation (single nucleotide deletion at nucleotide [nt] position 1081) in the *cpsE* gene that resulted in an early stop codon at amino acid 370, resulting in a severely truncated protein. Moreover, all of the capsular operon genes from *cpsF-cpsM* were deleted in these isolates (Table 3).

New South Wales, Australia

A similar but separate case occurred at a recirculating aquaculture farm in NSW: barramundi were vaccinated with autogenous bacterins prepared from isolates QMA0155 / QMA0220 that caused an outbreak that ran from late 2005 into early 2006. In November 2008, an outbreak occurred in vaccinated stock. As with the SA case, mortalities were low and associated with spine deformities in which streptococci were able to be detected in histology and from which *S. iniae* was subsequently isolated in culture. These isolates had a similar frameshift mutation in *cpsE*, coupled with deletion of *cpsF-cpsM* (Table 3).

Changes in genotype associated with vaccine failure are phenotypically and antigenically relevant

In the vaccine failure case in Northern Territory, Australia, a single mutation was found in the *cpsG* gene (from ST1 to ST3). A buoyant density assay was performed to assess any phenotypic variation between the *cps* genes. Buoyant density was different in the two isolates, with isolate QMA0177 traveling further through a continuous Percoll gradient than strain QMA0191. This occurred with multiple independently prepared cell suspensions through Percoll gradients prepared at two dilutions. In the case of the SA and NSW cases with truncated *cpsE* and major deletions in the operon, the differences in buoyant density were more pronounced, with the mutated isolates QMA0253, QMA0236, and QMA0249 migrating completely through the continuous gradient when prepared using diluted Percoll (Fig. 2).

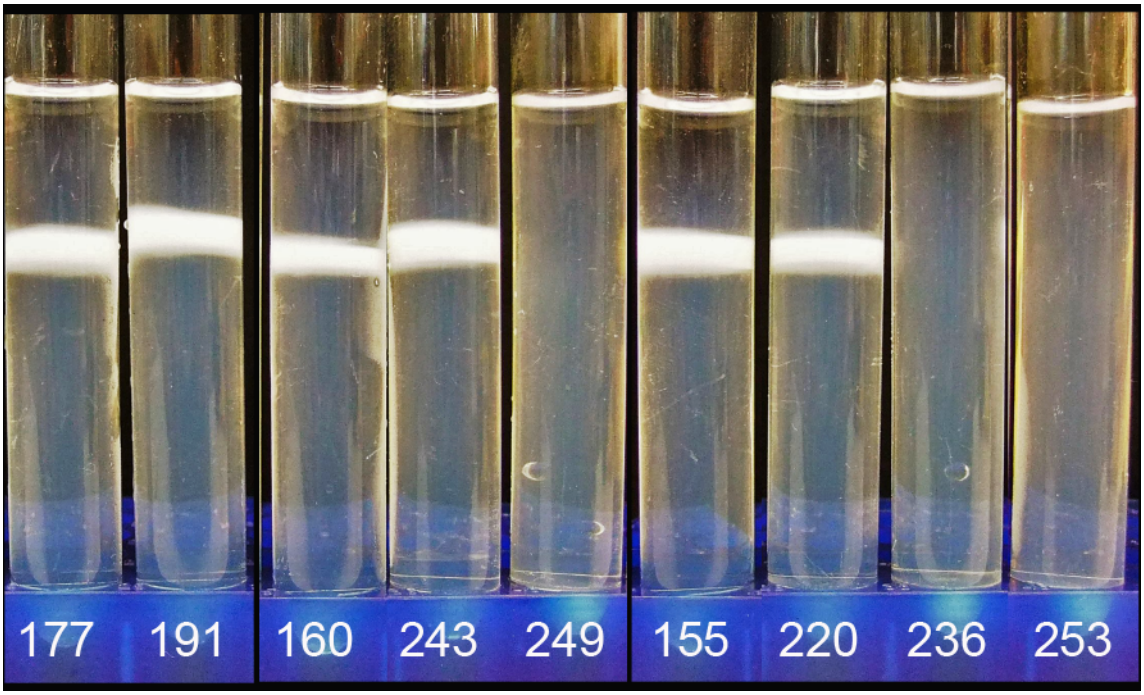

FIG 2 Percoll buoyant density assay for NT (QMA0177, QMA0191), SA (QMA0160, QMA0243, QMA0249), and NSW (QMA0155, QMA0220, QMA0236, QMA0253) isolates.

To investigate whether the changes seen in capsular operon that coincided with reinfection of vaccinated fish were antigenically relevant, antibodies were raised in barramundi against the two isolates from the index case of reinfection in NT. Antibodies raised against QMA0191 were poorly cross-reactive with QMA0177 by a whole cell ELISA (Fig. 3A). Similarly, antiserum raised against QMA0177 was poorly cross-reactive with QMA0191. In the case of the strains from NSW, antisera raised in barramundi against the initial vaccine isolate QMA0155 (same CPS sequence type as the subsequent vaccine strains QMA250 to QMA252) cross-reacted strongly with strain QMA0236, which occurred in vaccinated fish (Fig. 3B). When fish were vaccinated with QMA0236, a relatively low antibody response was detected in general, even against the same strain in a whole-cell ELISA, although it was higher than that for the negative control serum. Although the response was low, it was significantly higher in a whole-cell ELISA against the homologous strain than against QMA0155 (Fig. 3B)($p < 0.05$).

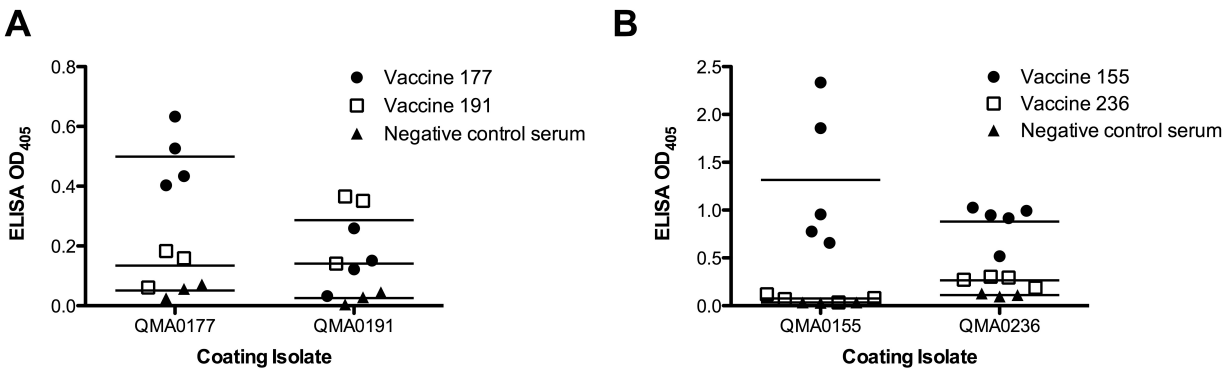

FIG 3 Antibody cross-reactivity by a whole-cell ELISA for selected isolates from the index case in NT (QMA0177, QMA0191) (A) and the NSW case (QMA0155, QMA0236) (B).

2.4 Discussion

Vaccination of finfish against bacterial pathogens has proven extremely effective at preventing mass mortality in farmed animals (29). However, it is this very efficacy that places a high selective pressure on pathogens and this may lead to dominance by new serotypes less well recognized by the resultant specific immune response to the original vaccine. In the present study, we investigated genetic changes in *S. iniae*, a fish pathogen with a track record of new serotype emergence in vaccinated animals (4). We found mutations in the capsular operon that coincided with infection in previously vaccinated stocks. These genetic changes likely lead to changes in polysaccharide biosynthesis (6, 11). Intriguingly, changes were restricted to a limited suite of genes within the ~ 20-gene operon. Firstly, two sequence types were found in *cpsY*. Of 39 isolate sequences, only strains from the Northern Territory, Australia, contained the *cpsY* ST2 variant, with all other isolates being identical to the type strain. It is intriguing that a new sequence type should only be found in NT, especially when movements of fingerlings interstate are common. This suggests that *S. iniae* infections arose locally in the fish after they were shipped and the bacterium was not cotransported with the fingerlings from the Darwin hatchery. *cpsY* ST2 would currently appear to be a robust diagnostic indicator for NT origin of the strain. *cpsY* is a transcriptional regulator that has an important role in intracellular survival in neutrophils, and is also essential for successful dissemination to the brain (2, 3). The functional role of the mutation in the present case is unknown, but isolates were recovered from the brain in both outbreaks.

cpsA through *cpsC* were always identical to the Type strain, regardless of country of origin. In *S. pneumoniae cpsD* is a self-phosphorylating tyrosine kinase, acting with *cpsB* and *cpsC* to regulate activity of *cpsE* (22-25). *cpsD* was found to vary in *S. iniae*, and was the

only coding mutation in the cps operon that was different between the two post-vaccine isolates from the Israeli case of vaccine failure, with a switch in the amino acid at position 123 from proline to leucine. While the effect of this mutation at the functional level remains to be determined, a proline-leucine switch at this position will substantially change the shape of the protein, removing a bend at this residue. The Israeli team reported that these two isolates (named KPF177 [QMA0188] and KPF404 [QMA0186]) were serologically similar when tested with rabbit antisera using Ouchterlony immunodiffusion, but they differ in RAPD profiles (4). Each of these strains has been used in autogenous vaccines and a novel serotype arose which differed in EPS production (11). It would be interesting to investigate the CPS operon of the newly emerged strain to determine whether coding mutations have arisen in the capsular biosynthesis machinery that led to increase polysaccharide over-expression, or failure to anchor the membrane. In Australia, a coding mutation in *cpsD* was never found as the sole mutation in the cps operon with most isolates analysed having the ST3 *cpsD* sequence type. The only other *cpsD* variant found in Australia isolates was *cpsD* ST1 and this was only found in association with a frame shift mutation in *cpsE*. Once again this *cpsD* mutation (ST3 to ST1) involved the proline-leucine switch at aa 123, derived from an identical single nt mutation. In Australia, however, the proline-leucine switch was also accompanied by a valine-leucine substitution at aa 175. The effects of *cpsD* mutations will be difficult to interpret in Australian isolates due to the universal accompaniment of the change with a frame shift in *cpsE*. In *S. iniae*, *cpsE* encodes a UDP-glucose-dependent glycosyl transferase that initiates synthesis and export of the capsular polymer. There was more diversity of this gene than any other of the cps genes, with 6 different sequence types found among the *S. iniae* isolates recovered in Australia, and 8 in total among the 39 isolates analysed. It is likely that mutations in this gene change the amount of capsular polysaccharide produced and this can have profound effects on the tissue distribution of the organism during disease (18). Indeed, the frame shift mutations that resulted in early termination of the *cpsE* gene that occurred in strains infecting vaccinated fish in NSW and SA substantially increased the buoyant density, suggesting reduced/eliminated capsular polysaccharide production. This was associated with lower mortality and completely transformed pathology and symptoms. While these mutations often co-occurred with deletion of genes *cpsF* through *cpsM* (isolates QMA0233-236; isolate QMA0249), it appears that the frame shift mutation in *cpsE* alone is sufficient to cause the phenotypic changes as isolate QMA0158 (frame shift in *cpsE*, but remainder of the operon intact) exhibited similar increase in buoyant density to the strains carrying the deletion of *cpsF-M*. Deletion of *cpsF-M* has been reported previously in a

“commensal” strain *S. iniae* (18). Our research suggests that these strains are not commensal, as they still cause pathology and mortality in a farm situation, but are somewhat attenuated (data not shown). This association with spinal fractures in barramundi is interesting as human spinal osteomyelitis caused by *Streptococcus dysgalactiae* subsp. *equisimilis* has been reported (14). The structure of teleost fish bone is without a marrow cavity and Haversian system, which means that osteomyelitis cannot occur. However, inflammation can develop in avascular tissue by extension from the adjacent periosteal vessels and generally takes the form of rarefaction (demineralization) rather than frank necrosis. Teleost bone usually cannot be immobilized in a fracture situation and thus will heal in a deformed fashion, as observed in the cases reported here (data not shown).

cpsG is a putative UDP-glucose 4 epimerase that converts UDP-glucose to UDP-galactose. Three variants of *cpsG,* involving an insertion or deletion of three amino acids, LSK, at aa positions 165, 166, and 167 in the ST1 type strain, were found. In ST2, these three amino acids were deleted, while in ST3, they were repeated, resulting in a protein that was three amino acids longer than the type strain. The phenotypic effects of these mutations remain to be determined, but mutations in this gene that alter enzyme efficiency may change the rate of conversion and thus change the ratio of glucose/galactose in the final capsular polymer, with resulting effects on surface epitopes. Mutation from *cpsG* ST1 to ST3 was the only change in the index case of vaccine failure in Australia. This involves a 3-aa LSK repeat insertion in ST3 compared to ST1. How this may affect function is yet to be elucidated, but the two isolates involved were serologically different, as determined by an ELISA using fish antiserum. Moreover, there was a small change in the buoyant density of the cells, suggesting a change in CPS structure. Future work will focus on expressing these variants as recombinant proteins and measuring their epimerase activity. This, coupled with LC-MS analysis of CPS from these two isolates, may elucidate how these mutations translate into antigenic variation. *cpsG* was deleted in a number of isolates, including the Israeli and Thai isolates, suggesting that it may not be essential for *cps* biosynthesis but may play an important role in the final molecular shape of the polysaccharides.

No cases of reinfected vaccinated fish have yet been associated with changes in *cpsH*, although two variants were found among the Australian strains. *cpsH* is a putative repeat unit polymerase, and changes in efficacy may change polymer length and, therefore, the final structure and antigenic nature of the capsule.

When disease arises in enclosed tank-based aquaculture systems, theoretically, best practice would be to close down the system, disinfect it completely, dismantle and clean pipework, pumps and filtration, then restart with clean stock. In practice this is not possible as multiple size-classes are generally kept within the same system to allow continual supply of table-sized fish for customers. Thus, outbreaks of *S. iniae* may be controlled with careful antibiotic treatment, and vaccinated stock may be reintroduced into the system. However, this means that the reservoir of infection is never really eradicated, providing ample opportunity for reinfection should immunity become compromised. In the closed systems in NSW and SA, careful use of autogenous vaccines initially controlled disease. In the latter period of the study, divalent vaccines were formulated after sequence typing to include the *cps* STs from the history of infection on the farm. It is intriguing that streptococcosis once again occurred in these farms in 2008/2009 but with substantially changed pathology and greatly reduced mortality among the infected fish. Moreover, strains from both sites were typed and found to carry the frameshift mutation in *cpsE* (*cpsE* ST5 / 5A) and the deletion of *cpsF* through *cpsM,* although fingerlings came from different sources. The development of these very similar cases suggests the possibility of a common origin for the isolate. However, a silent mutation after the early termination signal in *cpsE* was found in the NSW isolates (hence *cpsE* ST5A), hinting that the SA case and NSW case may have arisen independently. Interestingly, the unusual truncation of *cpsE* was also found in a strain isolated several years earlier in Thailand. Whether this type has been introduced or whether it is a natural adaptation to host specific-immunity that occurs independently is unknown. It is likely that such a serious mutation in the capsular operon results in complete cessation of the capsular biosynthesis machinery and therefore creates a capsule-deficient mutant, as evidenced by our buoyant density results. This may explain the unusual pathology and very low mortality rate in the fish, as a capsule deficient strain, while not recognised by anti-CPS antibodies, would be highly susceptible to phagocytic attack and may seek refuge in the bone of already-compromised fish. When antibodies raised against QMA0155, the initial sequence type found on this farm, were used in a whole cell ELISA, they cross-reacted with strain QMA0236 but not vice versa. This may reflect lack of cps in QMA0236; while whole-cell bacterins prepared with QMA0155 would result in anti-*cps* antibodies along with a range of antibodies against other cell components, including integral membrane proteins, which are highly conserved among *S. iniae* (6) and would be exposed to antibody binding on CPS-deficient QMA0236, the converse would not be true. Integral membrane proteins of QMA0155 would likely be coated with CPS and unavailable for antibody binding in a whole-cell ELISA.

In summary, coding mutations in the capsular operon of *S. iniae* appear to correlate with reinfection of previously vaccinated fish. These mutations are restricted to a few key genes in the ~20-gene operon. Two key questions remain to be answered. First, how do the genetic changes in the capsular operon, translated changes in the protein machinery for polysaccharide biosynthesis and export, actually lead to phenotypic and antigenic changes on the surface of the cell? Second, is vaccination driving mutation of highly mutable loci within a single strain type extant on a farm at a particular time, or is vaccination merely selecting the fittest variant from a pool of varied serotypes that are present among the population? The high ratio of nonsynonymous to synonymous mutations (dN/dS) among the genes of the CPS operon suggests that these changes are being driven under strong selective pressure. Further work aimed at answering these questions is ongoing.

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Roles of Personnel

ACB conceived and designed the project, performed much of the bioinformatics analysis and drafted the manuscript. CM re-analysed sequence data, prepared figures and co-wrote the manuscript. JCFB conducted the sequencing, including primer design and preliminary sequence analysis. ML, RC and SB provided the veterinary and pathology reports and supplied many of the strains. RC conducted the histopathology. CC, BY and FA conducted the antibody and phenotypic assays.

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Streptococcus iniae cpsG alters capsular carbohydrate composition and is a cause of serotype switching in vaccinated fish

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Abstract

Background: *Streptococcus iniae* causes septicaemia and meningitis in marine and freshwater fish wherever they are farmed in warm-temperate and tropical regions. Although serotype specific, vaccination with bacterins (killed bacterial cultures) is largely successful and vaccine failure occurs only occasionally through emergence of new capsular serotypes. Previously we showed that mutations in vaccine escapes are restricted to a limited repertoire of genes within the 20-gene capsular polysaccharide (*cps*) operon. *cpsG,* a putative UDP-galactose 4-epimerase, has three sequence types based on the insertion or deletion of the three amino acids leucine, serine and lysine in the substrate binding site of the protein.

Results: To elucidate the role of *cpsG* in capsular polysaccharide (CPS) biosynthesis and capsular composition, we first prepared isogenic knockout and complemented mutants of *cpsG* by allelic exchange mutagenesis. Deletion of *cpsG* resulted in changes to colony morphology and cell buoyant density, and also significantly decreased galactose content relative to glucose in the capsular polysaccharide as determined by GC-MS, consistent with epimerase activity of CpsG. There was also a metabolic penalty of *cpsG* knockout revealed by slower growth in complex media, and reduced proliferation in whole fish blood. Moreover, while antibodies raised in fish against the wild type cross-reacted in whole cell and *cps* ELISA, they did not cross-opsonise the mutant in a peripheral blood neutrophil opsonisation assay, consistent with reported vaccine escape.

Conclusions: We have shown here that mutation in *cpsG* results in altered CPS composition and this in turn results in poor cross-opsonisation that explains some of the historic vaccination failure on fish farms in Australia.

3.1 Introduction

Aquaculture currently supplies more than half of the finfish consumed annually and is now recognised as a major contributor to future food security against a background of continuing human population growth (1). However, the rapid growth of finfish aquaculture has encountered a number of challenges to environmental and economic sustainability including high losses from infectious diseases (2, 3). Vaccination of fish in aquaculture has transformed the control of mass mortality from bacterial diseases since the introduction of water-in-oil emulsion injectable vaccines in the 1990s, resulting in reduction in antibiotic use to negligible levels in industries where vaccination is routinely applied (4, 5). Widely employed for more than 20 years in the cold and cool-temperate water salmonid aquaculture industries, vaccination by injection is being adopted in warm-temperate and tropical aquaculture species where industry expansion is currently most rapid and novel disease challenges arise (6). In most cases, water-in-oil emulsion injectable fish vaccines provide lifetime protection through the farm cycle and protective efficacy has remained stable through many years of repeated use to vaccinate each new year-class of animals stocked into farm sites (5). Occasionally, however, reinfection of vaccinated fish does occur. For example, in salmonids, a novel biotype of *Yersinia ruckeri* emerged independently in rainbow trout in Europe and the USA that was not fully covered by existing *Y. ruckeri* vaccines (7-11). In warm water aquaculture, protection against *S. iniae* is also type-specific with novel capsular serotypes emerging relatively rapidly in vaccinated stock (12-14). The occurrence of vaccine-escape among streptococcal fish pathogens after the introduction of vaccination into these rapidly developing warm-water industries is potentially problematic, but not without precedent: Serotype switching in *Streptococcus* species is frequently associated with reinfection and vaccine escape in humans and terrestrial animals (15-18). In human infants, both serotype replacement and serotype switching have occurred at low prevalence since the introduction of polyvalent pneumococcal vaccines in the early 2000s (16, 19, 20). The molecular mechanisms behind serotype change are not completely understood, however non-synonymous point mutation in *wciP* has been attributed to capsular polysaccharide (cps) serotype switching in serotype 6A pneumococcus in vaccinated infants in Japan (21), and mutational analysis has attributed nucleotide changes to differences in polysaccharide composition (22, 23). In *S. iniae,* mutations in several genes within the ~20 gene *cps* operon have been associated with vaccine escape (14). In-frame deletion or insertion of nine nucleotides in the *cpsG* locus has been most frequently associated with vaccine escape during the autogenous vaccination cycle (14).

The capsular operon of *S. iniae* has been sequenced and annotated previously and *cpsG* was identified *in silico* as a UDP-galactose-4-epimerase (24). Epimerases play an important role in bacterial exopolysaccharide biosynthesis in both Gram-negative (25-27) and Gram-positive bacteria (28, 29) in which they catalyse the interconversion of hexose sugar isomers, such as glucose and galactose, often as a conjugate with a nucleotide diphosphate and/or a lipid carrier (30). UDP-galactose 4-epimerase from *E. coli* interconverts UDP-glucose and UDP-galactose via a hydrogen transfer pathway in which a complex between the enzyme and a diphosphopyridine nucleotide (which is reduced in the process) forms the UDP-4-keto sugar as an intermediate (31, 32). This cyclical pathway can proceed in either direction, dependent upon substrate availability and removal of the end product into other pathways. As a result of this bidirectional capability, predictions of how the epimerase may influence the final polysaccharide capsule structure are difficult, however epimerase activity is directly correlated with the amount of exopolysaccharide produced by *S. thermophilus* (33, 34). Therefore one might postulate that a reaction favoured in one direction over the other may result in dominance of either glucose or galactose in the final polysaccharide structure.

Given non-synonymous mutations in *S. iniae cpsG* are correlated with reinfections occurring in vaccinated fish, we examined the role of *cpsG* in *S. iniae* capsular polysaccharide (CPS) biosynthesis to determine how mutation of this gene may contribute to serotype switching and consequent vaccine escape.

3.2 Materials and Methods

Bacterial strains and culturing

Streptococcus iniae isolates collected from fish farms by veterinarians between 1995 and 2014 were stored as master seed stocks at -80°C in Todd-Hewitt broth (Oxoid) containing 20% glycerol until required and are listed in Table 1. Case histories are as described previously (14). Strains were recovered from stock without defrosting and grown on Columbia Agar base containing 5% defibrinated sheep blood (Oxoid) at 28°C for 24-48 h. *S. iniae* genomic DNA was isolated as described previously (35), and *S. iniae* identity and purity was confirmed by diagnostic PCR (36, 37), and by sequencing the 16S rRNA gene (36, 38). All strains were grown in Todd-Hewitt Broth (THB) overnight at 28°C, with gentle shaking, unless otherwise stated.

Table 1. Bacterial strains used in this study

Strain	*cpsG* ST	Comment	Reference
QMA0142	1	Isolated from non-vaccinated fish	14
QMA0177	3	Isolated from vaccinated fish	14
QMA0248	2	Isolated from vaccinated fish	14, 43
QMA0395	KO	*cpsG* knockout from QMA0142	This study
QMA0502	1	Rescue ST1 knock-in QMA0395	This study

In silico analysis of *cpsG*

Amino acid sequences for ST1, ST2 and ST3 variants of *cpsG* were analysed by BLAST using the conserved domain database (CDD) on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to identify putative active sites, cofactor binding sites and the substrate-binding site. Amino acid sequences were also compared using Provean to predict potential effects of the insertion/deletion mutations on CpsG enzyme activity (39). Molecular weights and pI were determined with ProtParam (http://web.expasy.org/cgi-bin/protparam/protparam).

In-frame allelic exchange mutagenesis

Wild type *S. iniae* isolate QMA0142 containing the ST1 variant of *cpsG* was used for allelic exchange mutagenesis. Primers used for PCR were based on the *cpsG* gene from the *S. iniae* capsular operon found in GenBank accession number AAY17299.1. For allelic exchange, primers were designed to contain a region ~ 500 bp upstream and downstream of the *S. iniae cpsG* gene sequence, and adjacent primers to *cpsG* were designed containing ~ 25-bp 5' extensions that parallel the 5' and 3' sequence of the spectinomycin

resistance (*aad9*) gene KC747117.1 (Fig. 1, Table 2). Phusion PCR reagents (New England Biolabs) were used to amplify upstream, downstream and complete spectinomycin amplicons, and to generate one fusion fragment, which was then cloned into pCR-Blunt-II (Invitrogen) to generate pCR-Blunt-ΔcpsG. The pCR-blunt-ΔcpsG construct was linearized by incubation with *sma*I and 10 μg of linearized DNA used to transform *S. iniae* isolate QMA0142 following a modified protocol (40), with incubations at 30°C and spectinomycin (100 μg/ml) used for selection of allelic replacement mutants. For complementation of *cpsG*, a fusion construct was designed using *cpsG* ST1 amplified from strain QMAO142 with the addition of a constitutive promoter, ribosome binding site (RBS), and the antibiotic resistance gene chloramphenicol acetyltransferase (*cat*) from pACYC (41) downstream of the *cpsG* gene (Fig. 1). Cloning and transformation was performed as above, except that chloramphenicol (4 μg/ml) was used for selection. Precise allelic exchange was confirmed by sequencing (Australian Equine Genetic Research Centre, Brisbane), and by PCR and RT-PCR as follows: Genomic DNA and RNA were purified from 5 mL mid-exponential THY cultures of WT QMA0142, mutant QMA0395 and rescue QMA0502 using DNeasy and RNEasy mini kits (Qiagen) according to the manufacturers protocol except that *S. iniae* cells were lysed with acid-washed glass beads (115 μm) for 50s at 7000 rpm in a Roche MagnaLyser. For cDNA synthesis, genomic DNA was digested from the RNA extractions on-column with the RNAse-free DNAse set (Qiagen) and the eluted total RNA was quantified by Qubit spectrophotometry before 300 ng RNA per strain was reverse transcribed using SuperScript ViLo (Invitrogen). RT-PCR was performed with 2uL cDNA as template using *cpsG, cpsF* and *cpsH* primer pairs (Table 2, Figure 1). Negative controls comprised non-transcribed RNA template with *cpsF* primer pair. Positive control was WT QMA0142 genomic DNA with each of the *cpsF, G* and *H* primer pairs.

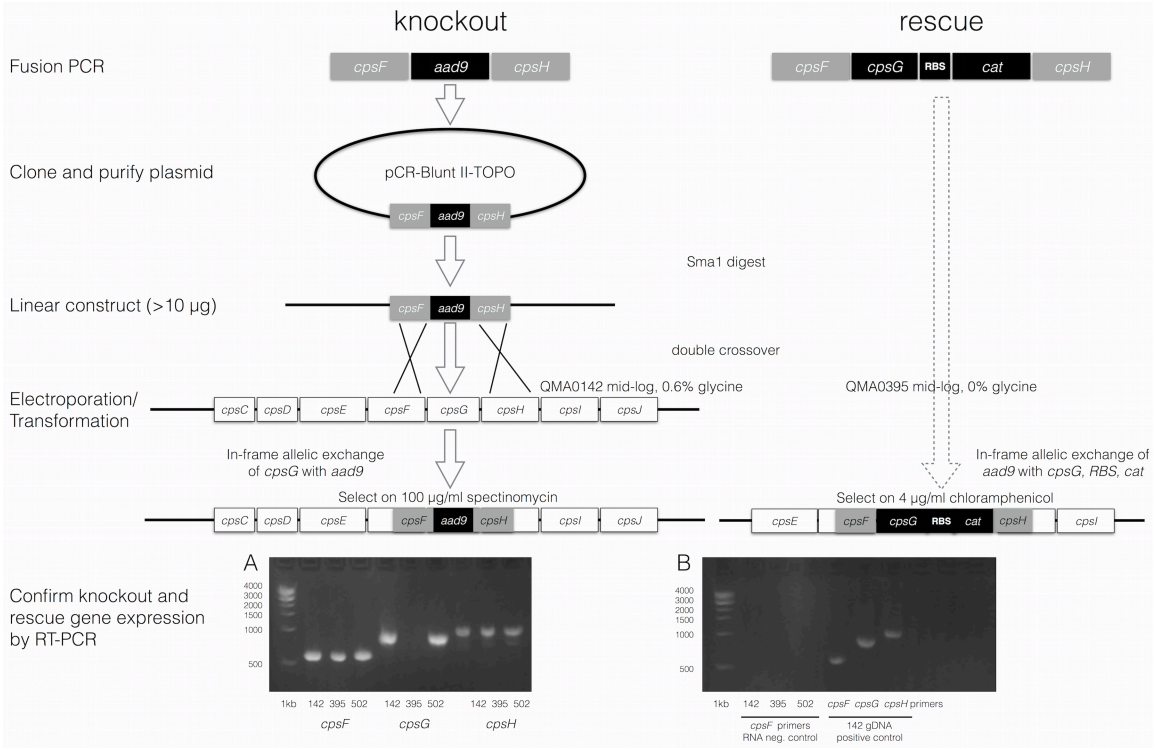

Figure 1. Schematic representation of in-frame allelic exchange mutagenesis for deletion and rescue of *cpsG* in *S. iniae* QMA0142. Knockout and rescue was confirmed by RT-PCR: A) cDNA template from 300ng total RNA extracted from QM0142 (WT), QMA0395 (mutant), QMA0502 (rescue), amplified with *cpsF* (lanes 2-4), *cpsG* (Lanes 5-7) and *cpsH* (lanes 8-10) primers. B) Control amplification using non-reverse transcribed RNA as template with *cpsF* primers (negative control, lanes 2-4) and genomic DNA from QMA0142 (WT) with *cpsF*, *cpsG* and *cpsH* primers (positive controls, lanes 5-7). NEB 1kb markers are included in lane 1 in both gels.

Table 2. Primers used for in-frame allelic exchange and protein expression

Primer	Sequence (S, sense; A, antisense)	Purpose
Primers for allelic replacement of cpsG		
aad9 S	5'cgtggaatcatcctcccaaa3' (S)	spectinomycin gene amplification
aad9 A	5'cactgcatttcccgcaatatc3' (A)	spectinomycin gene amplification
cpsGFlankup	5'gcatggatccgacccgtctgccatgataac 3'(S)	upstream sequence of *cpsG*
cpsGFlankdown	5'ctgcgaattccggtctaaagttaaatcctcgc 3' (A)	downstream sequence of *cpsG*
cpsGfusionUp	5'cgtatgtattcaaatatatcctcctcttttctcacttctccttattttgctctggtcc 3'(A)	upstream sequence of *cpsG* +*aad9*
cpsGfusionDown	5'taactataaactatttaaataacagatttagaagtgtgttacaagggaaaa aaattc 3'(S)	*aad9* + downstream sequence of *cpsG*
cpsGintDown	5'cgatgctcctgctgaaatgccaactc 3' (S)	downstream internal *cpsG*
cpsGintUp	5'gagttggcatttcagcaggagcatcg 3' (A)	upstream internal *cpsG*
Primers for complementing cpsG		
compG1	5'caatagtcattttatgatttacaaatttagaagc 3'(S)	upstream primer of gene before *cpsG*, *cpsF*
compG2	5'ctatatttttttcattgtttctttccgatattcg 3'(A)	end of *cpsG*
compG3	5'cggaaagaaacaatgaaaaaatatagcgattcaaaataggagacattt taaag 3'(S)	RBS + *cpsG*
compG4	5'ctttaaaatgtctcctattttgaatcgctatatttttttcattgtttctttccg3' (A)	RBS + *cpsG*
compG5	5'catttcaattgttcgcatcg 3' (A)	*cpsH* downstream
compG6	5'tggttaggagaaaaataaatgaaaaaatatagtagaagtgtgttac 3' (S)	*cat* + *cpsH*
compG7	5'tatcggaaagaaacaatgaaaaaatatagcgattcaaaataggagac attttaaag 3' (S)	*cpsG* + RBS
compG8	5'gtaacacacttctactatatttttttcatttatttttctcctaaccatgtctc 3' (A)	end of *cat* + *cpsH*
catF	5'gaggattggaatagaaagccata 3' (S)	*cat* amplification
catR	5'ttatttttctcctaaccatgtctca 3' (A)	*cat* amplification
Primers for RT-PCR confirmation of expression in mutants		
cpsF F	5'atgtatccttatattaaacgac 3'(S)	
cpsF R	5'tcacttctccttattttgctctg 3' (S)	complete *cpsF*
cpsG F	5'atgaaaaaagtacttattacaggtgc 3' (S)	
cpsG R	5'ctatatttttttcattgtttctttcc 3' (S)	complete *cpsG*
cpsH F	5'atgaaaaaatatagtagaagtgtg 3' (S)	
cpsH R	5'ttattcatcatcctgtttaatccctaaagta 3' (A)	complete *cpsH*

Capsular polysaccharide (CPS) extraction and hydrolysis

For CPS extraction, bacterial isolates were grown in 10 ml of THB overnight (Oxoid), and 0.5 ml was inoculated into 45 ml of fresh THB and grown overnight at 28°C. Cells were pelleted by centrifugation at 3,220 x *g* for 20 mins at 4°C (Eppendorf 5518E), and resuspended in 1 ml 150 mM Tris-HCl (pH 7.0), 1 mM $MgSO_4$. The suspension was

transferred to a 1.5 ml microcentrifuge tube and centrifuged (Eppendorf MiniSpin) at 12,100 x *g* for 10 min at room temperature. After decanting, the pellet was resuspended with 1 ml 150 mM Tris-HCl (pH 7.0), 1 mM $MgSO_4$ and 0.1% (w/v) of sodium deoxycholate, and incubated for 15 min at 37°C. After incubation, 10 U/ml mutanolysin (Sigma), 100 µg DNaseI (Roche) and 100 mg RNaseA (Invitrogen) were added and the mixture was incubated for 18 h at 37°C. After incubation, 100 µg Proteinase K (Sigma) was added and incubated for 4 h at 56°C. The suspension was centrifuged for 30 min at 12,100 x *g*, 4°C. The supernatant was transferred to a new 1.5 ml centrifuge tube and frozen at -80°C for 48 h, lyophilised overnight, and hydrolysed. Hydrolysis was performed using 2M TFA (trifluoroacetic acid) in 10 mg of CPS, at 80°C for 4 h, centrifuged for 10 min at 12,100 x *g*, at room temperature. The supernatant was filtered (0.22 µm), and the filtrate was used for GC-MS.

Gas chromatography mass spectrometry (GC-MS)

Aliquots (100 µl) of each hydrolysed sample were freeze-dried. Derivatization and introduction of the samples was then performed by an autosampler (MPS-2XL) equipped with a heated agitator (Gerstel Mülheim an der Ruhr). Each sample was derivatized to its Meox-TMS derivatives through 1 h reaction with 10 µl of 40 mg/ml methoxyamine hydroxychloride solution in pyridine (Sigma-Aldrich, St Louis, MO, USA), followed by 2 h reaction with 20 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% Trimethylchlorosilane (TMCS) (Machery-Nagel), both at 37^{o}C under constant stirring at 750 rpm. Derivatized samples (1 ul) were then injected in splitless mode at 250^{o}C using helium as a carrier gas under a constant flow of 1 ml/min. Metabolites were separated on a Varian capillary column (Factor FOUR VF-5 ms: 0.25 mm, 0.25 µm, 30 m length with a 10 m fused guard column) (Varian) installed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C MSD mass spectrometer (Agilent, Mulgrave, VIC, Australia). The initial temperature of the separation program was held for 1 min at 70^{o}C, then increased to 325^{o}C at a rate of 7^{o}C /min and held for 3.5 min. The ion source, quadrupole and transfer line were set at 300^{o}C, 150^{o}C and 280^{o}C respectively. Glucose and galactose were detected in both total ion count mode (TIC) and in selected ion monitoring mode (SIM) for identification and quantification purposes respectively. Identification was achieved by matching the retention times and the fragmentation patterns to standards (Sigma-Aldrich, St Louis, MO, USA). Relative quantification was achieved by dividing the peak area of the characteristic ion of glucose to galactose with mass to charge (*m/z*) ratio of 319.

Growth curves for QMA0142, Δ*cpsG* and complemented Δ*cpsG*

Starter cultures of bacterial isolates were prepared in THB grown overnight as above. A 2 %(v/v) inoculum was then used to inoculate fresh THB or terrific broth (Difco) with either D-glucose or D-galactose (to 55mM from a 10% stock solution) added as a carbon source in a 96-well flat-bottomed plate (Greiner). Growth was measured by measuring optical density at 600 nm, over a 12 h period at 28°C. Assays were performed in triplicate with independent starter cultures each time.

Vaccine formulation and antibody production

Killed bacterin vaccines were prepared by picking a single colony from QMA0142 and Δ*cpsG* grown overnight on blood agar and following established protocols (42). The vaccines were formulated as emulsions in Freund's incomplete adjuvant delivered by intraperitoneal injection as described previously (42). Eight juvenile barramundi (*Lates calcarifer*) were vaccinated per treatment, including PBS/adjuvant emulsion controls. Blood was collected from euthanized (overdose of anaesthetic, Aqui-S) fish after 900 degree days (30 days at 30°C), by caudal venipuncture. Blood was allowed to clot for 1 h at room temperature, then overnight at 4°C to ensure complete removal of fibrin and other clotting factors. Serum was collected by decanting the supernatant after centrifugation at 12,100 x *g* for 5 min and stored at -20°C until required. Animal use was approved by the UQ Animal Ethics Committee (AEC) for project SBS/060/10 "Protecting farmed barramundi from Streptococcal infection by vaccination".

Detection of CPS antigenic cross-reactivity by ELISA

Serum collected from injected fish was used in whole cell and CPS extract ELISA following a modified protocol (42). Capsule extracts were prepared as described above, and high-binding 96-well ELISA plates (Greiner) were coated with 2 µg CPS from each strain in 100 µl carbonate bicarbonate buffer.

Whole blood bactericidal and opsonisation assays

Fresh blood was collected by caudal venipuncture from barramundi (*Lates calcarifer*) euthanized by anaesthetic overdose. Blood bactericidal assays were performed essentially as described previously (43). Bacteria were enumerated before and after incubation for 60 min in blood by viable counts of 25 µl aliquots on TH agar plates in replicates of 6, following incubation overnight at 28°C. For opsonisation, bacteria were incubated for 2 h in serum at a concentration previously determined by ELISA, then incubated with blood for 60 min to determine survival. Blood for bactericidal assays was obtained by coordinated tissue sharing with UQ AEC approved project SBS/056/13 "Cellular immunity in fish:

Robust defence against infection or Achilles' heel?" whereby euthanized fish had to be bled prior to recovery of monocytes from haematopoietic tissues (44).

Transmission Electron Microscopy (TEM)

Mid-exponential cultures (10 ml) of each strain were prepared by using overnight THB cultures as starter inocula (2% v/v) in fresh THB and incubating for ~5 h at 28°C with gentle agitation. Cells were washed in 70 mM sodium cacodylate buffer and immediately fixed on ice in glutaraldehyde diluted to 3.6% in 70 mM sodium cacodylate buffer for 1 h. Fixative was removed with 3 washes in cacodylate buffer and the cells were stained with ferritin (0.1 mg/L) in cacodylate buffer for 1 h on ice. Cells were washed 6 x in 70 mM cacodylate buffer then processed for TEM using standard dehydration and embedding processes.

Statistical Analyses

Data analyses were performed with Prism 6 (GraphPad Software, Inc.). Growth curves were fitted using the Weibul equation and then compared by F-test. *In vitro* whole-blood opsonisation and bactericidal activity data were analysed by ANOVA followed by Tukey's multiple comparison tests post hoc. ELISA data were analysed by two-way ANOVA to identify significant effect of vaccine and coating antigen. In all cases $p = 0.05$ was employed to define statistical significance.

3.3 Results

Phenotypic effects of *cpsG* deletion in *S. iniae*

To further elucidate the roles of *cpsG* in CPS biosynthesis and serotype switching an isogenic knockout was prepared in the *cpsG* wild type strain of *S. iniae* QMA0142 isolated from barramundi in Northern Territory, Australia during a disease epizootic. Knockout of *cpsG* was confirmed by RT-PCR showing normal mid-log expression of *cpsF, cpsG* and *cpsH* in WT and rescue mutant, but no expression of *cpsG* in the knockout (Fig. 1). Moreover, similar expression of *cpsH* downstream of *cpsG* in the knockout and rescue mutants compared with the wild type parent suggested no polar effects of the in-frame allelic exchange in the operon (Fig. 1). Wild type isolates of *S. iniae* QMA0142 develop small umbonate translucent colonies on sheep blood agar overnight at 28°C whereas the *cpsG* mutant produced small white opaque colonies (Fig. 2A). Buoyant density of the cells was marginally affected by the *cpsG* mutation with cell suspensions becoming more diffuse in a Percoll separation compared to the parent (Fig. 2B). To investigate possible metabolic effects from the mutation, growth curves in liquid culture of the parental

QMA0142, *cpsG* mutant strain and complemented mutant were compared. There were significant differences ($p < 0.001$, F-test) between growth curves for the *cpsG* mutant and the wild-type parent strain as the *cpsG* mutant grew more slowly in all three growth media tested (Fig. 2D-E). Growth was fully restored in the complemented mutant (Fig. 2D-E).

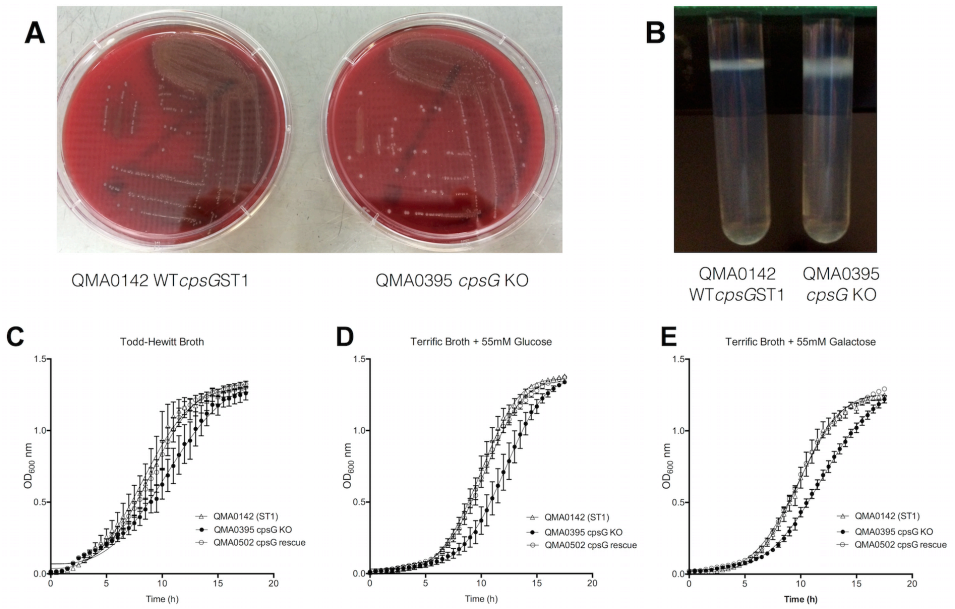

Figure 2. Phenotypic traits of QMA0142 wild type *cpsG* ST1 and QMA0395 *cpsG* knockout prepared from parent QMA0142 by allelic exchange. A) Colony morphology on Columbia agar with 5% defibrinated sheep blood. B) Buoyant density in continuous Percoll gradients. C) Growth of wild type parent QMA0142, D) *cpsG* knockout mutant QMA0395 and E) complemented *cpsG* rescue QMA0395 in different growth media.

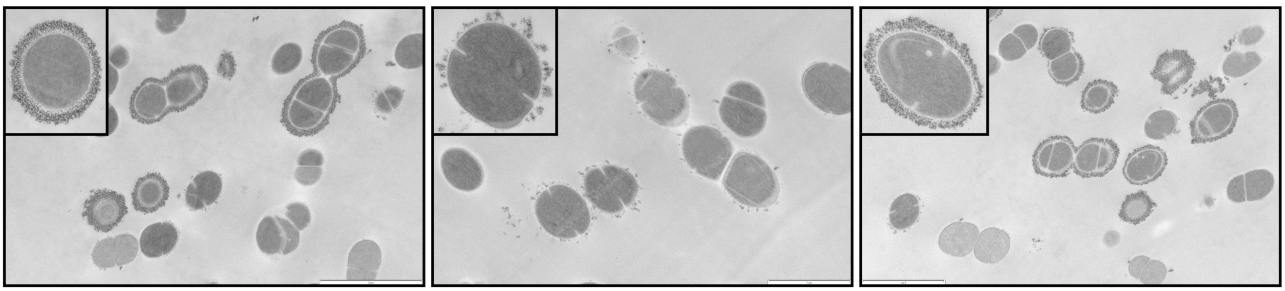

Figure 3. Transmission electron micrographs showing cells of QMA0142 (WTST1), QMA0395 (*cpsG* KO) and QMA0502 in which has been rescued by knock-in of *cpsG* into QMA0395. Cells stained with 0.1% ferritin to label capsular polysaccharide. Scale bar in main picture = 1 µm. Insets show high magnification of single cells.

There was an apparent ultrastructural change in CPS when stained with ferritin and observed by TEM (Fig. 3). The ferritin binding was less dense and less uniform across the cell surface in the knockout mutant compared to either the WT or the rescued mutant (Fig.

3 insets). However, there was variability in CPS cell coverage between cells within each culture when viewed under TEM at lower magnification, with some cells having no apparent attached capsule (Fig. 3). ST1 wild type and the rescued mutant exclusively exhibited complete surface coverage with CPS, which was viewed under TEM (Fig. 3).

Deletion of *cpsG* increases levels of capsular glucose relative to galactose

GC-MS was used to quantify glucose and galactose in CPS extracts from parental QMA0142, *cpsG* mutant and complemented mutant and wild type *cpsG* ST variants ST2 and ST3. CPS from Δ*cpsG* had significantly increased glucose content compared to the wild type ST1, whereas the capsule purified from the complemented mutant contained levels of glucose similar to those of the WT (Fig. 4). CPS from the ST3 variant contained a marginal increase in glucose relative to galactose compared with ST1, while CPS extracted from ST2 comprised the highest levels of glucose out of all the samples tested (Fig. 4). To determine whether the variation in glucose/galactose ratio in CPS in the different sequence types could be explained by the insertion deletion mutations, the amino acid sequences were analysed for effect on epimerase activity in Provean. Provean predicted a deleterious effect of the deletion of LSK in ST2 compared to ST1 (score -14.556, Fig 5), and also insertion of an additional LSK in ST3 relative to ST1 (score –9.803, Fig 5).

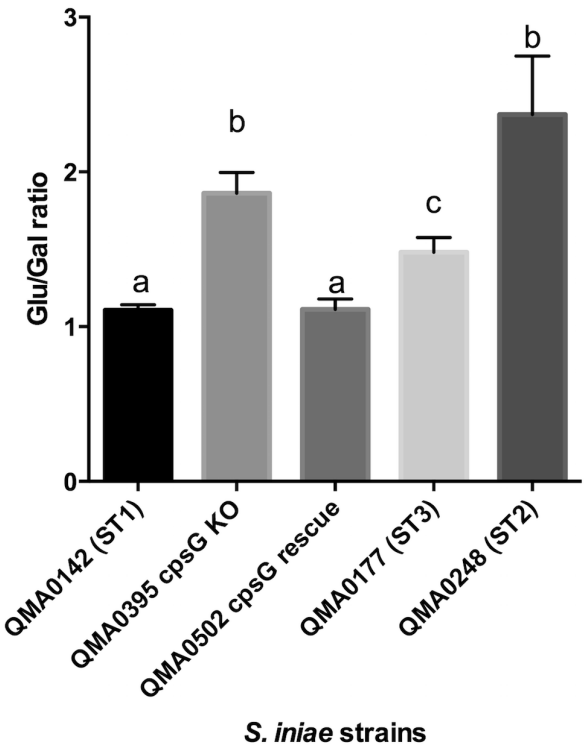

Figure 4. Glucose: Galactose ratio in purified hydrolyzed CPS determined by GC-MS. Differing letters indicate statistically significant differences (Unpaired *t*-test $p<0.05$)

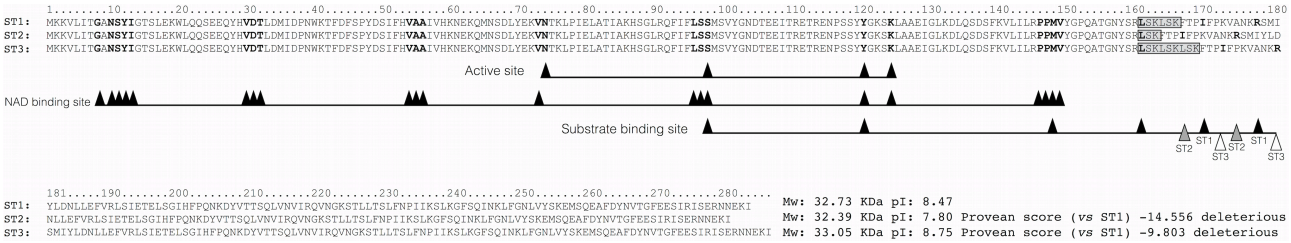

Figure 5. Amino acid sequences of ST1, ST2 and ST3 variants of *cpsG* from *S. iniae* showing active sites, NAD binding sites and substrate binding site predicted *in silico* by conserved domain database search. Insertion and deletion mutations in the variants are indicated by shaded boxes. Box inset indicates molecular weights, pI and effect of insertion and deletion mutations on enzyme activity predicted by Provean (39).

Role of *cpsG* in immunogenicity and cross-opsonisation in fish

To determine how changes in *cpsG* effect antibody recognition, juvenile barramundi were immunised with formalin killed bacterins prepared from WT ST1 parental strain QMAO142 and the *cpsG* deletion mutant, and a virulent ST2 variant, QMA0248, that has been shown to be virulent in barramundi and illicit protective immunity by vaccination in barramundi (42). In whole cell ELISA, neither QMA0142 nor Δ*cpsG* elicited a strong antibody response compared to adjuvant only controls (Fig. 6A). This was not due to any problem with the fish as the positive control vaccine against QMA0248 elicited a significant specific homologous antibody response compared to the adjuvant control (Fig. 6A). As *S. iniae* M-protein can bind host immunoglobulin in a non-immune manner via the Fc portion of the IgM molecule (45, 46) potentially masking specific immune binding in whole cell reactions, a second ELISA in which purified CPS was employed as the coating antigen was performed. This revealed that there was specific antibody response against CPS, but there was no significant difference between sera from fish injected with the knockout compared with sera from fish injected with the wild type CPS (Fig. 6B). To determine differences in the ability of the respective antisera to opsonise *S. iniae,* the wild type, *ΔcpsG*, and complemented mutant were pre-incubated with normal serum (control), or antiserum against wild type QMA0142 (ST1) or QMA0395 (*ΔcpsG*), prior to inoculation into fresh whole blood from barramundi. Non-opsonised cells grew significantly during the 1 h incubation, increasing cell numbers by more than 50% for all strains except *ΔcpsG* where the increase in cell number was less than 10% over 1 h. Opsonisation with anti-QMA0142 antibody resulted in significant killing of QMA0142 (Fig. 6C). Growth of the *ΔcpsG* mutant was not significantly reduced by either anti-QMA0142 or anti-*ΔcpsG* antibody in the whole-blood killing assay.

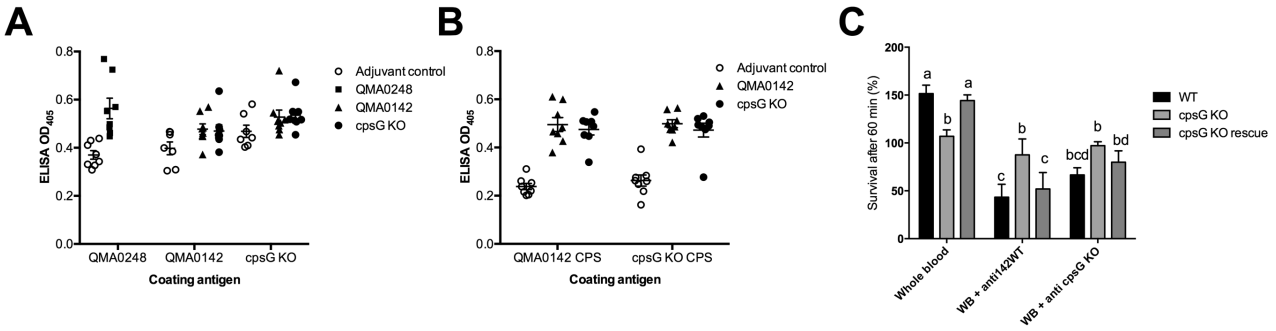

Figure 6. Immunoreactivity of wild type and *cpsG* knockout mutant *S. iniae* by whole cell (A) and purified CPS (B) ELISA. C) Opsonisation by barramundi antibodies in whole barramundi blood bactericidal assay. Differences between treatments with differing letters are significant (ANOVA with *post-hoc* Tukey's multiple comparison tests $p<0.05$).

3.4 Discussion

The capsular polysaccharide is a major virulence factor in *S. iniae*, and CPS-deficient knockouts are attenuated (43). Nonetheless, wild-type CPS deficient isolates have been identified in naturally infected farmed fish, albeit causing markedly different pathology and greatly reduced morbidity (14). CPS promotes virulence by enhancing survival in fish blood possibly by inhibition of phagocytosis (43). However, there is a trade-off as CPS is a major antigen recognised by the fish adaptive immune system (47), consequently vaccination with formalin killed bacterins is highly protective against homologous CPS-types (42, 48, 49). Introduction of vaccination against *S. iniae* in several aquaculture species has resulted in relatively rapid emergence of novel capsular serotypes and consequent vaccine failure (12-14). There is no defined serological typing system for *S iniae* but vaccine escapes are antigenically distinct (12), have differing exopolysaccharides (13) and are correlated with coding mutations in a limited subset of the 20 genes of the *cps* operon (14). In Australian barramundi farms, killed monovalent or polyvalent autogenous vaccines are used that include the strains isolated from the most recent infections (14). Here, insertion/deletion mutations in *cpsG* occur most frequently in vaccine escapes.

The *cpsG* gene encodes a putative UDP-galactose-4-epimerase (24) and epimerase activity is supported here as the capsular polysaccharide of our *cpsG* knockout mutant comprised substantially more glucose compared to galactose than the wild type parent strain, suggesting that epimerase activity is favoured in the glucose to galactose direction. Insertion/deletion mutations in the substrate binding site region gene found in naturally occurring vaccine escape strains also resulted in changes in the glucose/galactose ratio of

the capsule by GC-MS. This was consistent with *in silico* predictions of the effects of these mutations using Provean, in which both the insertion (ST3) and the deletion (ST2) mutations resulted in predicted deleterious effect on activity. The deletion had a greater effect than the insertion (Fig. 4); resulting in a 50% and 100% increase in glucose relative to galactose in the variant CPS compared ST1 CPS (Fig. 3).

Higher glucose in the capsule marginally impacted buoyant density in cells, evidenced in Percoll gradients and this correlated with reduced ferritin binding observed in TEM. However, there was considerable variability in electron dense surface structure revealed by ferritin within the same culture. This may reflect differing capsular expression levels between individual cells as capsular polysaccharide biosynthesis has been shown to be phase variable in other species such as *S. pneumoniae* and *N. meningitides* (50, 51) There was a significant affect on growth in blood from naive barramundi: *S. iniae cpsG* ST1 wild type strain QMA0142 increased 50% in blood after 1 h. In contrast, the *cpsG* mutant did not proliferate in blood during the same period. Similar differences in proliferation/killing determined in *ex vivo* blood survival assays have been reported between CPS types I, II and IV of *S. pneumoniae* (17). It is possible that the differing capsular composition in several Streptococcus species increases visibility to lectin and/or neutrophil detection thereby reducing the ability to proliferate in whole blood. However it is more likely that the effect detected in *S. iniae* in this study is metabolic, as growth in Todd-Hewitt broth and terrific broth with either glucose or galactose as the preferred carbon source was also impeded in the *cpsG* knockout compared to wild type, and was fully restored in the complemented mutant. This suggests that this epimerase, in spite of its membrane location, may be co-opted into general carbon metabolic pathways in *S. iniae,* consistent with efficient use of genetic resources by bacteria and continued evolution by genome reduction. Indeed, epimerase from the Leloir pathway has been implicated in CPS production in some strains of *S. thermophilus*, perhaps through biosynthesis of essential precursors as epimerase activity is higher in strains with no galactose in the CPS (52). Ongoing analyses of wild type and *cpsG* mutant isolates may better inform how this epimerase may be used in energy metabolism.

When measured by whole cell ELISA, antibody response was low against both the *S. iniae* wild type and the *cpsG* mutant in barramundi. Fish yield a much lower detectable antibody response to vaccination, in terms of titre, than mammals. This reflects the generally lower affinity and avidity of teleost immunoglobulins (predominantly tetrameric IgM in serum, with some monomeric IgT in some species) and the lack of immunoglobulin class-switching

(53). The high background in the whole cell ELISA indicated by high optical density detected with sera from adjuvant only controls is likely due to non-immune binding of the serum immunoglobulins by the SiMA M-like proteins in *S. iniae* (46). This is partially corroborated by the decrease in background and increase in resolution between vaccinated and non-vaccinated fish detected when CPS was employed as the coating ELISA antigen. It is also possible to increase resolution in whole cell ELISA with *S. iniae* by using 2% normal goat serum as a blocking agent instead of BSA (42). Nevertheless, there was antigenic cross-reactivity between the knockout and wild-type isolate in the anti-CPS ELISA, but this did not translate to cross-opsonisation as the heterologous sera failed to opsonise the bacteria in whole blood. Cross-reactivity but low cross-opsonisation has also been reported in patients vaccinated against *S. pneumoniae* (54, 55) and has been attributed to low avidity of IgG after class-switching (55). However, it is difficult to draw parallels as the B-cell response in fish is different to that in humans and class switching is thought not to occur, although responses are considered very complex and are not yet fully understood (53). Nevertheless, opsonising antibody is likely to explain the very high protection reported for killed bacterins against *S. iniae* in fish (42). Lack of cross-opsonisation between different *cps* types in *S. iniae* is the probable explanation for occasional vaccine escape recorded in fish farms (12, 14).

3.5 Conclusions

We have shown here that functional mutations in *cpsG* encoding an epimerase in the capsular operon of *S. iniae* result in altered CPS composition and this in turn results in poor cross-opsonisation that might explain some of the historic vaccination failure on fish farms in Australia. The activity of epimerases in switching monosaccharide isomers and thereby affecting structural phenotype of polysaccharides downstream in the biosynthesis pathway makes them likely candidates for strong selection by protective host immunity directed against the polysaccharide structure. These findings have implications for the understanding of vaccine escape in other pathogens of humans and animals where epimerases are critical key steps in the biosynthesis of major serotype-specific antigenic polysaccharides including capsule and lipopolysaccharide. Further work is required to determine whether these changes in the *cps* operon arise spontaneously by mutation, are selected in response to stress imposed by the immune host, are acquired horizontally, or are in fact a result of serotype replacement from a pool of extant strains in the farm environment that have comparatively increased fitness in the presence of immunised fish.

Competing interests

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Authors’ contributions

ACB and MJW conceived and designed the study, CMH and CMG created the knockout and knock-in mutants. CMH conducted all phenotypic and immune assays. ACB conducted additional control PCR/RT-PCR and prepared samples for TEM. PC conducted the GC-MS. CMH and ACB drafted the manuscript and prepared all figures. All authors edited the final draft.

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Chapter 4

Conclusions & Future Directions

“Somewhere, something incredible is waiting to be known.”
-Carl Sagan

4. Conclusions & Discussion

The ability of bacteria to evolve between or during infection in response to clinical intervention requires in-depth understanding of the pathogen and its interactions within the host (1, 2). The molecular genetics of host pathogen interactions are shedding light on metabolic pathways that contribute to pathogen survival and the vast datasets provided by genome re-sequencing are highlighting evolution of potential vaccine targets during use (3). Epitope evolution and antigenicity is a major concern for developers of vaccine candidates (4-6), including streptococcal CPS designed vaccines (7, 8).

The objective of the work described in this was to better understand molecular evolution of the capsular operon, the role of mutation in specific capsule biosynthesis genes, and the affect of such mutation on capsular antigenicity of *Streptococcus iniae*. To address this objective three aims were employed:

1) ***Identify re-emerging strains of S. iniae and identify genetic changes using large scale screening of capsular genes***

2)*. **Use allelic replacement to knockout genes in the capsular operon of S. iniae, and elucidate phenotypes with a defined genetic background***

3) ***Raise antibodies in barramundi and use ELISA and bacterial killing assays to determine antigenic properties of mutant strains***

Chapter 2 addressed the first aim in an investigation of re-emerging strains of *S. iniae* in previously vaccinated barramundi (*Lates calcarifer*) on fish farms in Australia. The capsular operon of 39 strains was genetically screened, identifying several mutations, but only in a limited repertoire of genes in the operon. Of the 21 kb region screened, mutations occurred within what is thought to be the biosynthesis region of the operon (*cpsY, cpsD, cpsE, cpsG & cpsH*). A unique amino acid arrangement was found in some strains of *cpsG* where LSK (leucine, serine, lysine) was deleted in strain type 2, and the same amino acid sequence duplicated in strain type 3. Amino acid changes can lead to antigenic variation and antimicrobial resistance in a diverse range of bacteria (9, 10). Since *cpsG* is postulated to be an epimerase, a critical enzyme for interchanging glucose and galactose, we hypothesized that these amino acid changes would ultimately affect the antigenicity of the CPS. Additionally, eight strain types were identified for *cpsE* composed of non-synonymous mutations and a frame-shift mutation in one of the re-emerging strains. Subsequent histology of the infected barramundi revealed *S. iniae* in the bone and

acapsular forms. The mutations in the biosynthesis genes and new pathology discovered in *S. iniae* provided the foundation for the rest of this thesis.

Chapter 3 addressed aims 2 and 3 using microbiological and molecular approaches. With specific biosynthesis genes being identified in Chapter 2, as well as mutations within those genes, experiments were undertaken to understand how these identified genes function in the capsular operon. *cpsG*, encoding a UDP-glucose 4'-epimerase, was genetically knocked-out to ascertain effects on capsule production, and ultimately how fish host immune cells would perceive modified *S. iniae*. Since cpsG is thought to regulate the glucose:galactose ratio for capsule production, we hypothesised that by deleting the *cpsG* gene there would be a measureable difference in *S. iniae* capsular production and the immune responses of fish. The gene was successfully knocked-out and found to act on the glucose:galactose ratio in capsule production. Immunoassays revealed that the mutant did not grow well in whole blood and cross-opsonisation was less than for other strains tested. Additionally, in an ELISA assay, detection by barramundi antibodies was minimal and no noticeable difference was observed when comparing the mutant to the WT. We concluded that the increase in glucose in the mutant might be allowing the mutant to go undetected, and that the amino acid mutations in *cpsG* (as in those found in chapter 2) are affecting the glucose:galactose ratio and could be contributing to re-emergence in the field.

Molecular biology techniques have greatly improved the current understanding of genetics and virulence of streptococci. Employing genetic techniques such as allelic exchange are being used to provide insight on gene function, virulence, and antigenicity (9, 11, 12). Previous research has focused on biosynthesis genes in the CPS operon of several pathogenic bacteria, investigating function and contributing to better vaccine design (13-16). The majority of these studies highlight the contribution to virulence or lack thereof for bacterial CPS biosynthesis genes using various animal models including zebra fish (*Danio rerio*) and mice (17, 18). In order to appropriately assess virulence it is necessary to challenge a model organism and look for attenuated or accelerated potential of the pathogen in question. Another approach before considering virulence testing would be to evaluate mutational effects on antigenicity. This should be considered in cases where the pathogen in question has re-emerged after a vaccine program (19). Many vaccines are frequently reformulated due to evolution of pathogens after clinical intervention (4, 7, 20, 21). Investigating these highly antigenic targets is critical for vaccine design and for understanding evolution of pathogenic bacteria (22).

This thesis was conceived following the collection of data from re-emerging *S. iniae* strains from multiple barramundi fish farms around Australia. Re-emerging *S. iniae* strains not only occurred in Australia, but also in Israel after vaccination (19), highlighting the need to understand effects of clinical intervention on fish farms. Previous research on *S. iniae* infections in fish has shown various pathology and virulence mechanisms (23-25), yet understanding how re-infection occurs remains less well known.

Since several Australian fish farms were experiencing massive barramundi losses due to infections with *S. iniae* it was imperative to investigate the main vaccine target in *S. iniae*, the CPS. We undertook this task by performing a genetic screen of the approximately 21 genes present in the CPS operon. Other groups have performed similar experiments with the pneumococci to investigate serotype changes (13). Similarly, we found several mutations in key biosynthesis genes.

Several genes have been thoroughly described in the CPS operon of *S.iniae*, including *cpsD* (autophosphorylating protein tyrosine kinase) and *cpsY* (transcriptional regulator), providing key information on how the bacteria regulate CPS production (14, 16), but we found these genes to be highly conserved amongst pre and post vaccination isolates and therefore unlikely to contribute to serotype shift in these cases. However, no previous studies had investigated the sugar-modifying components of the operon that are most likely to have an antigenic role such as *cpsG* in *S. iniae*. Given the unique situation of having an essentially "closed system" through the cyclical autogenous vaccination programs, I was able to provide insight on epitope evolution after vaccination. This information paved the way for investigating the other mutated genes identified in the screen, with particular focus on *cpsE*, which revealed a further eight distinct strain types due to non-synonymous mutations.

A glycosyltransferase (GT), *cpsE*, is thought to be critical in the capsule biosynthesis process, specifically for building the polysaccharide (26). The utilization of GT's varies between organisms, but in Gram-positive and Gram-negative bacteria they are located in the ABC transport pathway (Gram negative) and Wzy and synthase dependent pathways (Gram positive) (26). These pathways are responsible for synthesis and export of polysaccharide to the cell surface to produce an extracellular capsule or cell wall structure that assists the bacterium in evading the host's immune system (27). Genes that encode GT's in bacteria such as *S. iniae* and *Streptococcus pneumoniae* are located in the CPS operon (15, 28). Mutations in GT's have led to capsule variability and altered antigenicity

in *S. pneumoniae* (29). GT mutations also impact Gram-negative bacteria: in *Salmonella enterica* and *Salmonella bongori,* heterogeneity of the O-antigen of the lipopolysaccharide (LPS) occurs via genetic recombination events in glucosyltransferase (same family as glycosyltransferases) genes that are located outside of the LPS operon (30).

In Gram-positive *Streptococcus thermophilus*, the GT's are grouped together (*epsE*, *epsF*, *epsG* and *epsI*), with each one contributing to the production of the exopolysaccharide (31). Similarly, in the CPS operon of *S*. *iniae*, a group of putative GT's (*cpsE*, *cpsF*, *cpsK*, and *cpsL*) is located, yet it is unknown if these function together to construct the polysaccharide as in other streptococcal species (32). What we do know is that the genetic screen described in Chapter 2 revealed no mutations in the GT genes: *cpsF*, *cpsK* or *cpsL*, with mutations only identified in *cpsE* (8). Additionally, an unusual pathology was observed in one strain, where *cpsE* had a frameshift mutation resulting in an early stop codon and multiple downstream genes were deleted (including the other GT's). Buoyant density assays showed the strain was acapsular, and histology revealed the bacterium residing in the bone of the fish in contrast to infection of the blood, meninges and choroid rete that is normally associated with *S. iniae* pathology. None of the other strains obtained displayed this unusual pathology, except this strain type missing multiple biosynthesis genes from the CPS operon. One potential explanation for multiple synomymous and non-synonymous mutations in *cpsE* is that GTs downstream in the operon can effectively fulfil the same role. In contrast, when these subsequent highly conserved GT genes are missing CPS biosynthesis is effectively inactive. This is in keeping with a hypothesis that that the genome of *S. iniae* is undergoing reductive evolution, and that *cpsE* may eventually be lost from genome. Evolution in response to changing environmental niches has been documented in many pathogenic bacteria and genome reduction has been previously described (33, 34). Understanding the function of *cpsE* and the other GT's in *S. iniae* will help elucidate which genes are absolutely required for polysaccharide synthesis.

The findings presented here shed light on two genes in the CPS operon of *S. iniae* and their role in capsule biosynthesis. The effects on antigenicity were determined, albeit they did not alter the capsule in such a way that would make these mutant forms vaccine candidates. Instead, this work provided insight into the function of these CPS genes and their contribution to immune evasion. Multiple spontaneous mutations occurring in the CPS operon after vaccination highlight the capacity of *S. iniae* to respond evolutionarily to specific vaccines, and ultimately switch the serotype. Autogenous vaccines on Australian fish farms are driving epitope variation, and there is a continuing need to further

understand and monitor the genes contributing to antigenic variation in *S. iniae*. Moreover, the search for conserved essential surface or secreted factors to target in vaccines is more important than ever. With the rapid advance of bacterial genomics accompanied by greatly reduced cost, identifying such candidates is becoming both feasible and affordable for lower economic value species such as fish.

Future Directions

Recent advances in genomics have shed light on our understanding of pathogen dispersal and evolution. For instance, a recent study on outbreaks of *Escherichia coli* ST131, extrapolated when virulence factors were acquired and the overall effect on global dissemination (35). Additionally, virulence evolution has been elaborately characterised for entire bacterial genera such as Yersinia (36). Since the genome was recently sequenced for *S. iniae* (*32*), it is now possible to explore using these methods to investigate metabolic pathways of CPS biosynthesis genes in greater detail.

At the outset of this project in 2012, large-scale bacterial genomics was beginning to advance but was still expensive, and out of reach for research in the field of fish diseases. Over the last 4 years the costs have fallen dramatically bringing large-scale whole genome within reach for investigating evolution and dispersal of fish pathogens. For example, genomics revealed that the salmonid pathogen *Renibacterium salmoninarum* has evolved incredibly slowly as it has been dispersed through Northern Europe and North America (37). A small-scale genomic study on terrestrial and aquatic strains of *Streptococcus agalactiae* also revealed slow evolution amongst aquatic strains (38). Not only are researchers identifying evolutionary time scales, but they are also finding mechanisms for adapting to various environments. In *Aeromonas salmonicida*, a fish pathogen, Vincent and colleagues (2016) discovered a strain that was evolving from a mesophilic environment to a psychrophilic lifestyle. Their comparison of *A. salmonicida* strains with the rest of *Aeromonas* genus species found high amounts of diversity in *A. salmonicida* in which they postulated was due to insertion sequences (39). The application of genomics coupled to evolutionary analysis can greatly accelerate vaccine design and improvement through reverse vaccinology (3, 40). This approach has been successfully implemented targeting slowly evolving protein antigens in *Neisseria meningitidis* (serogroup B) found in humans (41). Reverse vaccinology can be used now to cost-effectively design and steward fish vaccines through the filed lifetimes, which will be critical as aquaculture grows to supply the world with sustainable fish.

4.1 References

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Appendix

S1. Table of *S. iniae* isolates used in this study, including country/ state and site of origin (where known), isolation date, and ST of variable genes from the capsular operon (after Barnes, 2010)(1).

Strain	*cpsY*	*cpsC*	*cpsD*	*cpsE*	*cpsG*	*cpsH*	State (site)	Previous Designation	Date
Type Strain									
QMA0140	1	1	1	1	1	1	USA	ATCC29178	1970
Australian Isolates (by state)									
QMA0072	1	1	3	3A	1	1	QLD(1)	*95-40786*	1995
QMA0076	1	1	3	4	1	1	QLD(2)	*98-49835*	1998
QMA0078	1	1	3	3	1	1	QLD(3)	*1-41291*	2001
QMA0165	1	1	3	3A	1	2	QLD(4)	06-40593	2006
QMA0170	1	1	3	3A	1	2	QLD(4)	06-43734	2006
QMA0164	1	1	3	3A	1	2	QLD(5)	06-40191	2006
QMA0216	1	1	3	7			QLD(6)	E	2007
QMA0218	1	1	3	3A	1	2	QLD(4)	G	2007
QMA0155	1	1	3	3	2	1	NSW(1)	1	12/2005
QMA0220	1	1	3	3	2	1	NSW(1)	I	08/2006
QMA0250	1	1	3	3	2	1	NSW(1)	I6	11/2007
QMA0251	1	1	3	3	2	1	NSW(1)	I7	06/2008
QMA0252	1	1	3	3	2	1	NSW(1)	I8	06/2008
QMA0233	1	1	1	5A*	X	X	NSW(1)	1	11/2008
QMA0253	1	1	1	5A*	X	X	NSW(1)	I9	01/2009
QMA0254	1	1	1	5A*	X	X	NSW(1)	I10	01/2009
QMA0236	1	1	1	5A*	X	X	NSW(1)	4	03/2009
QMA0191	2	1	3	3	1	1	NT(1)	05/0409	04/2005
QMA0142	2	1	3	3	1	1	NT(1)	05/0430-D	07/2005
QMA0150	2	1	3	3	1	1	NT(1)	05/1151-D	08/2005
QMA0153	2	1	3	3	1	1	NT(1)	05/1383-D	09/2005
QMA0177	2	1	3	3	3	1	NT(1)	06/0784-G	07/2006
QMA0158	1	1	3	6*	2	1	SA(1)	4	02/2006
QMA0159	1	1	3	3	2	1	SA(2)	5	02/2006
QMA0160	1	1	3	3	1	1	SA(3)	6	12/1999
QMA0173	1	1	3	3	2	1	SA(?)	AVM-2958	2006
QMA0243	1	1	3	3	2	1	SA(3)	R1	05/2006
QMA0244	1	1	3	3	2	1	SA(3)	R2	10/2008
QMA0245	1	1	3	3	2	1	SA(3)	R3	10/2008
QMA0246	1	1	3	3	2	1	SA(3)	R4	03/2009
QMA0247	1	1	3	3	2	1	SA(3)	R5	03/2009
QMA0248	1	1	3	3	2	1	SA(3)	R6	03/2009
QMA0249	1	1	1	5*	X	X	SA(3)	R7	05/2009
QMA0083	1	1	3	3	2	1	WA(1)	AS-04-0006#1	2004
International Isolates									
QMA0186	1	1	2	2	X	1	Israel	KFP404	
QMA0188	1	1	3A	2	X	1	Israel	KFP173	
QMA0189	1	1	4	8	X	1	Réunion Is.	21-96 (2)	1996
QMA0190	1	1	1	5*	1	1	Thailand	CII.5b-88	1988

QLD, Queensland, NSW, New South Wales, NT, Northern Territory. Separate farm sites are indicated by differing numbers in parentheses. Month and year of isolation are given where known. X indicates deleted gene. *frameshift mutation resulting in early stop codon

S2. Primers and annealing temperatures for amplification of variable *cps* genes in the capsular operon of *S. iniae* (after Barnes, 2010)(1).

Forward primer	Reverse primer	Gene/s targeted	Annealing temperature (ºC)	Expected product size (kb)
HKi F	CPSA R	*cpsY*	65	3.0
CPSB F	CPSD R	*cpsB-D*	55	2.2
CPSE F	CPSE R	*cpsE*	58	1.8
CPSF F	CPSH R	*cpsF-H*	64	2.5

S3. Oligonucleotide primer sequences used for sequencing capsular operon genes from templates generated by long-range PCR (after Barnes, 2010)(1).

Primer	Sequence 5'-3'
CPS Y F	TTATATTTCCTTTTTTTGTGTCAATTTGA
CPS Y R	ATGAGAATACAACAATTACATTACA
CPS A F	TGATTGGAGTTAAAAAGTAATG
CPS A R	ATCGCATAGGATGGCAATTCA
CPS B F	CAGTAATGGGAGGAAAGTAAATG
CPS C R	TTGTGACATCCTTAACCTC
CPS D R	TCACTTTCTGGAATGTTTTTTAC
CPS E F	ATGAAAAGAAGTCAAAAAAGAGTAATC
CPS E R	TTACTCCTGTTTAGCGTCATTTA
CPS F F	ATGTATCCTTATATTAAACGAC
CPS F R	TCACTTCTCCTTATTTTGCTCTG
CPS G F	ATGAAAAAAGTACTTATTACAGGTGC
CPS G R	CTATATTTTTTCATTGTTTCTTTCC
CPS H F	ATGAAAAAATATAGTAGAAGTGTG
CPS I R	AATCTCCTATTTTTTCCCACAT
CPS J F	ATGAAAATACTTGTGACAGGTG
CPS K F	ATGATAACTGTTTGTATGGCAAC
CPS K R	TTACCATTTTTTATTTTTCCTTTTTGTTT
CPS L F	ATGAAAGTAGCGTTTTATTTAGAT
CPS L F RC	ATCTAAATAAAACGCTACTTTCAT
CPS L R	GTGTTCATCTGGTATTTTTTATTAA
CPS L R RC	TTAATAAAAAATACCAGATGAACAC
ORF276 F	ATGAGGGTATCTATAAAAAGTATAA
ORF193 F	ATGTTGCTGTTACTGACATTAACATT
ORF193 F RC	AATGTTAATGTCAGTAACAGCAACAT
ORF193 i R	AAACTAGAACCTGAAAGAAAAATACC
ORF151 R	TCATAGATACTCCTTTTCCTGTT
CPS M F RC	AATTCAATTTTATACTGTTTTTTTTCAT
CPS M R	ATATAAGAACTAAAAAATTCAAGTCAAATTAA
CPS N F	ATGAAAAAAATAGCAGTTGCTGG
CPS N R	TTAATCCCGGCCGAAAAGGT
ORF183 R	TTAGACTTCCTGAGCTGCGAA

Reference

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